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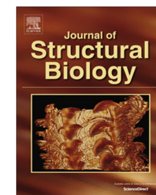

A new method for the separation and purification of the osteogenic compounds of nacre Ethanol Soluble Matrix

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ABSTRACT

Nacre is able to induce bone-forming cells mineralization, and gains widely interest in bone regeneration. While, the osteoinductive compounds are not yet identified. ESM (Ethanol Soluble Matrix), a nacre extract from powder of *Pinctada margaritifera* pearl oyster shell, has been firstly proven having the capacity to induce mineralization and to restore mineralization defect *in vitro*. It is suitable to treat ESM as a source of osteoinductive compounds. Herein, we develop a new method for separating and purifying nacre extracts by an ionic approach.

At first, cationic ESM (ESMc) and anionic ESM (ESMa) were achieved with ion-exchange resin. Then, ESM was separated and collected on cation exchange HPLC. Scanning Electron Microscopy coupled with Energy Dispersive X-ray Spectrometry (EDS) was used to reveal the concentrated elements in ESM fractions. A coupled cell models were used to test the ESM fractions. Alizarin Red staining was performed and quantified to evaluate the mineralization level.

ESMc and 2 HPLC fractions stimulated the mineralization in both cells. EDS demonstrated the abundant presence of calcium and chloride in the osteogenic fractions. To validate, pure $CaCl_2$ was tested and proven having an osteogenic effect in both cells, but less stable than ESM. The mineralization nodules induced by ESM fractions and $CaCl_2$ differed in both cells.

In conclusion, a new method was developed for separating and purifying nacre extracts by an ionic approach. By which, the osteoinductive compounds in ESM were proven cationic, and calcium in ESM was demonstrated to play a role in inducing the cell mineralization.

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1. Introduction

Nacre, or mother of pearl, is a calcified structure widely found in the internal layer of many mollusk shells, composed of aragonite (97%) and organic matrix (3%). Due to the worldwide availability, low cost, mechanical resistance, biocompatibility, biodegradability, opaque to X-rays and osteogenic feature (Chaturvedi et al., 2013), the natural material gains widespread interest in bone regeneration, as candidate for bone graft substitutes (Atlan et al., 1999, 1997; Meriem Lamghari et al., 2001; Rousseau et al., 2012), or in the case of pathologies affecting mineralization such as osteoporosis and osteoarthritis (Brion et al., 2015; M. Lamghari et al., 2001).

Nacre was demonstrated to be able to induce bone forming cells mineralization, by attaching directly osteoblasts (Atlan et al., 1997), or stimulating cells from bone marrow stromal cell to pre-osteoblastic cell toward osteogenic differentiation (Green et al., 2015; Milet et al., 2004), and was believed to contain some signal molecules in the organic matrix. The molecules could be released when nacre was implanted in bone environment, and induced osteogenesis (Atlan et al., 1997).

As reviewed, the organic matrix of nacre is a mixture of proteins, peptides, glycoproteins, chitin, lipids, pigments (Marin et al., 2012). To reveal the mechanism of nacre-driven mineralization, nacre extract was studied to identify the osteoinductive compounds. The most studied extract was WSM (Water Soluble Matrix). WSM from different species, were demonstrated osteoinductive in various cell models (Lopez et al., 2003; Milet et al., 2004). The nacre proteins were equally proven to play a primary role in controlling the formation of interfacial structure and biocompatibility with bone as well as the stability of biogenic tissues (Kim et al., 2002). While, it was also argued that the nacre molecules efficient in bone cell differentiation were probably more related to peptides (Rousseau et al., 2008; Rousseau, 2003). To

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date, more than 50 proteins and 50 peptides from nacre have been identified (UniProt protein database, see www.uniprot.org).

Though, the nacre osteoinductive compounds have not yet been identified. Ethanol extraction process for biomolecules has been involved previously (Huebner and Bietz, 1993; Esen, 1986; Colvin, 1961), but very recently, ESM (Ethanol Soluble Matrix), an organic extract from nacre powder of *Pinctada (P.) margaritifera* pearl oyster shell, has been firstly proven having the capacity to induce mineralization in mouse pre-osteoblastic cell line MC3T3-E1, and also the capacity to restore mineralization defect in human subchondral osteoarthritic (OA) osteoblasts (Brion et al., 2015). It is suitable to treat ESM as a source of osteoinductive compounds. For many years, the MC3T3-E1 cell line, deriving from newborn mouse calvarias (Wang et al., 2011), has an inherent potential to mineralize, and is widely used in the literature to mimic osteoblast behavior in the context of differentiation studies. While, human osteoarthritic osteoblasts present a mineralization defect (Couchourel et al., 2009). Thus, the coupled *in vitro* cell models are picky but practical for the validation of osteogenic effect.

To date, ESM seems to be a most active osteogenic matrix from nacre, and offers a good opportunity to identify the osteoinductive compounds. Herein, we developed a new method to separate and purify the matrix. The method is emphasized at an ionic approach, by using the ion resin to get a primary result and then ion-exchange HPLC as a secondary procedure to obtain the active molecules, toward the direction of identification. All the ionic fractions of ESM, prepared by ion resin or collected from HPLC, were tested onto the coupled cell models for confirming their osteogenic activity.

2. Material and methods

2.1. Nacre extract

Nacre powder was isolated from the *P. margaritifera* pearl oyster. ESM was extracted with ethanol as described previously (Brion et al., 2015). Precisely, nacre powder (250 g) was stirred with 500 ml of ethanol absolute anhydrous (CARLOERBA, Cat. 4127012) containing 0.1% HCl (VWR, Cat. 20252) for 24 h at 40 °C at 100 rpm by Rotavapor (Heidolph, Hei-Vap). The suspension was then centrifuged (20 min, 3000 rpm, 4 °C) and filtered (0.22 μm, Millipore) before being evaporated. The nacre extract was known as "Ethanol Soluble Matrix" (ESM).

2.2. Preparation of cationic ESM (ESMc), anionic ESM (ESMa) and ESME

Generally, the chemical extraction was done according to Fig. 1.

From the results of pre-tests by Alizarin Red staining, ESMc had an osteoinductive effect on OA osteoblasts, but not for ESMa. Then, we tried to recycle the active cations (ESME) from the cation exchange resin. That's why ESME was prepared, and no similar fraction from anion exchange resin was done simultaneously.

2.2.1. ESMc

100 mg ESM was weighed and solubilized in 50 ml water (milli-Q) to obtain a 2 mg/ml solution, then regenerated 50 ml anion exchange resin (SIGMA–ALDRICH, $DOWEX_{22}$ CL, Cat. 436623) by 200 ml 1 M NaCl in a conical flask and removed NaCl; The resin should be rinsed with 300 ml water (milli-Q) for 5 times in total and the washing water was removed each time; Then the ESM solution was added into the resin, and the flask was shaken manually to facilitate the ion-exchange before the exchanged solution being recycled; Afterwards, the resin was rinsed two times with 50 ml water (milli-Q), and the washed water should be pooled with the exchanged solution before being filtered (0.22 μm, Millipore), frozen at −20 °C and lyophilized to get ESMc.

2.2.2. ESMa and ESME

ESMa was extracted similarly as ESMc, but using cation exchange resin (GE Healthcare Life Sciences, SP Sepharose High Performance, Cat. 17–1087-01). As for ESME, the extraction followed closely the preparation of ESMa. After the lavation for getting ESMa, 50 ml 1 M NaCl was added into the resin, the flask was shaken manually to facilitate the ion-exchange before recycling the exchanged solution; The solution was frozen and lyophilized to get a dried mixture of NaCl and ESME; Then ESME was extracted from the mixture by 3 lavation of 100 ml ethanol absolute anhydrous (CARLOERBA, Cat. 4127012); The extracted solvent was filtered (0.22 μm, Millipore) and evaporated at 40 °C, 200mBar at 100 rpm by Rotavapor (Heidolph, Hei-Vap) to get ESME.

2.3. Separation and collection on cation exchange HPLC

2.3.1. Equipment of HPLC, column and general information for injection

The HPLC chain was products of WATERS. Interchim Column UP5SCX-250/046, a strong cation exchange column, was used for the separation of cationic molecules. For mobile phase, an ERLIC (electrostatic repulsion–hydrophilic interaction chromatography) method was adopted (Alpert, 2008), and developed. The column was conditioned with acetonitrile, and then in gradient mode, the phase mobile was followed the Table 1. The solvents at different channels were indicated as follows: Channel A, water (milli-Q); Channel B, ammonium acetate (MERCK, Cat. A135416836) 30% and acetonitrile (Sigma–Aldrich, Cat. 34851) 70%, pH 6.05; Channel C, acetonitrile (Sigma–Aldrich, Cat. 34851).

2.3.2. Separation and comparison of ESM, ESMc, ESMa and ESME

ESM, ESMc, ESMa and ESME were solubilized in water (milli-Q) respectively to get a solution at 10 mg/ml, and filtered (0.22 μm, Millipore). 100 μl of samples were injected, detected by a 2998 Photodiode Array detector (WATERS), and compared at 254 nm and 215 nm.

2.3.3. Collection of ESM

ESM was prepared at 25 mg/ml, 100 μl per-injection for the collection. The fractions were collected according to the profile of elution at 254 nm and 215 nm. The collection included the whole elution.

2.4. Scanning Electron Microscopy (SEM) coupled with Energy Dispersive X-ray Spectrometry (EDS)

Nacre powder and nacre extract were prepared onto a carbon coated metal device (specific for SEM), then the samples were desiccated and metalized. Metallographic observations were performed using a field emission gun scanning electron microscope (FEG-SEM JEOL7600F) equipped with an energy dispersive X-ray spectroscopy system (EDS-Oxford).

2.5. Cell culture and mineralization tests

This part was previously described (Brion et al., 2015) and adopted strictly. Some details and modification were indicated below.

2.5.1. Cell culture

Human osteoarthritic osteoblasts was achieved from the subchondral bone from OA patients undergoing total knee replacement (cooperation with Nancy Central Hospital, France), and

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