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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.

Key words: Nacre, Ethanol Soluble Matrix, MC3T3-E1 cells, Osteoarthritis osteoblasts, Biomineralization

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)(Atlan et al., 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 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-exchage HPLC as a secondary procedure to obtain the active molecules, towards 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 (250g) was stirred with 500ml of ethanol absolute anhydrous (CARLOERBA, Cat. 4127012) containing 0.1% HCl (VWR, Cat. 20252) for

24h at 40°C at 100rpm by Rotavapor (Heidolph, Hei-Vap). The suspension was then centrifuged (20min, 3000rpm, 4°C) and filtered (0.22µm, 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.

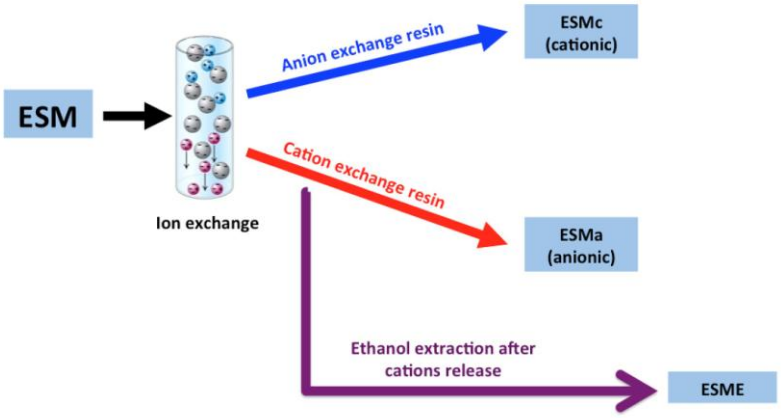


Fig. 1. *Schema for the preparation of cationic ESM (ESMc), anionic ESM (ESMa) and the losed cationic part (ESME) during the preparation of ESMa.*

2.2.1 ESMc

100mg ESM was weighed and solubilized in 50ml water (milli-Q) to obtain a 2mg/ml solution, then regenerated 50ml anion exchange resin (SIGMA-ALDRICH, DOWEX$_{22}$ CL, Cat. 436623) by 200ml 1M 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 shaked manually to facilitate the ion-exchange before the exchanged solution being recycled; Afterwards, the resin was rinsed two times with 50ml water (milli-Q), and the washed water should be pooled with the exchanged solution before being filtered (0.22µm, Millipore), frozen at -20°C and lyophilized to get ESMc.

2.2.1 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, 50ml 1M NaCl was added into the resin, the flask was shaked 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 100ml ethanol absolute anhydrous (CARLOERBA, Cat. 4127012); The extracted solvent was filtered (0.22µm, Millipore) and evaporated at 40°C, 200mBar at 100rpm 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 informations for injection

The HPLC chain were 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 10mg/ml, and filtered (0.22µm, Millipore). 100µl of samples were injected, detected by a 2998 Photodiode Array detector (WATERS), and compared at 254nm and 215nm.

Table 1

Mobile phase of elution program on cation exchange HPLC.

Time (min)	Flow rate (ml/min)	Channel A (%)	Channel B (%)	Channel C (%)
0	0.5			100
0.1	0.5			100
25	0.5		100	
40	0.5		100	
41	0.5	100		
71	0.5	100		
72	0.5			100
102	0.5			100

2.3.2. Collection of ESM

ESM was prepared at 25mg/ml, 100µl per-injection for the collection. The fractions were collected according to the profile of elution at 254nm and 215nm. 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 used at the 1st passage; mouse MC3T3-E1 cell line were provided from the European Collection of Cell Cultures (Sigma Aldrich, 99072810).

2.5.2. Treatment

ESM fractions and the identified compound were solubilized in culture medium and sterilized by filtration (0.22µm, Millipore) before the treatment. OA osteoblasts and MC3T3-E1 cells were seeded in triplicate onto 48-well plates at 14 000 cell/cm² and 25 000 cell/cm² respectively. 10% FBS DMEM/F12 and 10% FBS αMEM medium were respectively used. After adherence, the cells were treated with ESM fractions at 200 µg/ml (except noted as 100 µg/ml) for 7, 14, 21 days in OA

osteoblasts, and 7, 10, 12, 14 days in MC3T3-E1 cells. Ascorbic acid (50 µg/ml, Sigma) and β-glycerophosphate (10 mM, Sigma) were added into both culture media, used as control. Dexamethasone treated cells (100 nM, Sigma) were used as positive control.

2.5.3. Alizarin Red staining

The mineralization in extracellular matrix (ECM) was visualized by Alizarin Red staining, as the deposited calcium appeared red (Stanford et al., 1995). The stained cells were imaged with a LEICA microscope (M80, obj x1) to get a general view of mineralization or an observation of mineralization nodules in detail. To quantify the degree of mineralization, the stained cultures were destained and the Alizarin Red concentrations in each well were determined by absorbance measurement at 405 nm, as previously described (Gregory et al., 2004).

2.6. Statistical analysis

Data was presented as mean ± standard error of the mean for each condition. Each experiment was repeated independently three times (n=3). One factor-analysis of variance (ANOVA) was performed to evaluate the effect of each variable on two or more independent groups. For comparison between two group means, in which the response was affected by a single variable, an impaired t-test was performed. Any difference with a probability value less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. *Scanning Electron Microscopy reveals the ethanol extraction procedure as a moderate process for nacre extraction*

Previously, the mostly tested WSM, as a major type of nacre extract, was extracted by water without decalcification (Rousseau et al., 2008). While, it has been long to believe that the osteoinductive

compounds in nacre extract are referred to some organic molecules. Thus, it appears more reasonable to perform the extraction with an organic solvent, to avoid the interference of minerals and to get a higher concentrated organic matrix. The ethanol is chosen. As ESM is a very pioneering matrix for the research of nacreous osteoinductive compounds (Brion et al., 2015), it is necessary to confirm that the new procedure is also of no-decalcification.

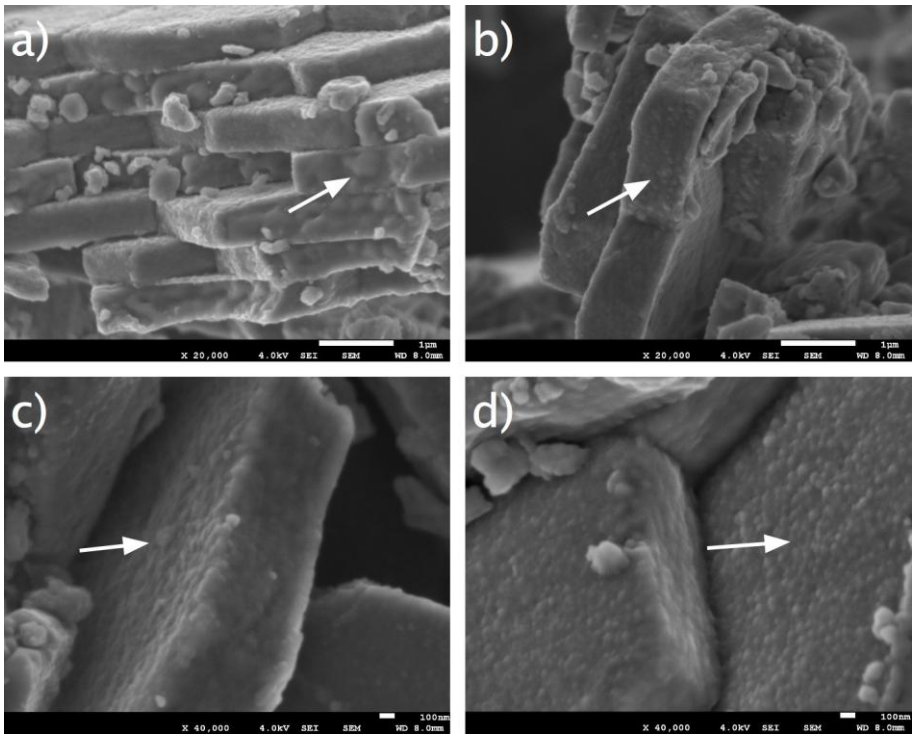


Fig. 2. *SEM micrographs of the pre-extraction (a, c) and post-extraction (b, d) nacre powder, SEI mode, 4kV. The organic matrix at lateral surface (arrow in a and b) and frontal surface (arrow in c and d) of nacre tablet is extracted by ethanol (b and d), without modification to nacre micro-architected structures. Scale bar corresponds to 1 µm (a, b) and 100 nm (c, d) respectively.*

For this purpose, the pre-extraction and post-extraction nacre powders are well desiccated, then observed under SEM. The observation is in SEI (secondary electron in-lens) mode at 4kV. Before (Fig. 2a and c) or after the extraction (Fig. 2b and d), the characteristic brick-and-mortar structure of nacre is always sustained, and no trace of modification to the microstructure is observed. In addition, at the same scale, the organic matrix at lateral surface (arrow in Fig. 2a) and frontal surface (arrow in Fig. 2c) of nacre tablet decreased obviously (arrow in Fig. 2b and d), leaving a much smoother surface

for the post-extraction powder. Directly and indirectly, the observation suggests that the ethanol extraction is a moderate process, which takes place only at the exposed surface and non-invasion into the crystal tablet, confirming that the process is without decalcification.

3.2. ESM is separated and purified as ESMc, ESMa and ESME by using ion-exchange resin

To investigate the osteoinductive compounds in ESM, we try this time an ionic approach. The first question to respond is that the osteoinductive compounds are cationic, anionic or neuter. Thus, as described above, the cations and anions in ESM are removed respectively by using ion-exchange resin to get anionic ESM and cationic ESM. From the results of pre-tests by Alizarin Red staining (data not shown), ESMc has an osteoinductive effect on OA osteoblasts, but not for ESMa. So, it is supposed that the osteoinductive compounds are cationic, and cation exchange resin has effectively caught the cations in ESM, which makes ESMa inactive for the osteoinduction in OA osteoblasts. To validate this hypothesis, it is no better than recycling the active cations from the cation exchange resin. That's why ESME is prepared, and no similar fraction from anion exchange resin is done simultaneously.

When the extractions are completed, i.e., after the lyophilization, the powders are scraped to pool into a glass flacon, and some optical micrographs are performed *in situ*. As shown in Fig. 3, generally, ESM, ESMc and ESME, which contain cations (excepting for Sodium), share a green appearance, while ESMa appears canary, totally different from the other ones. Compared to ESM, the 3 fractions have a more uniform color, implying that the complexity of ESM is decreased after a second extraction with water, as the substences which are indissolvable into water have been removed during the filtration. Besides, ESMc and ESMa look much finer than ESM, which could be regarded as a sign of purification. While, in ESME, many chunks are easily observed, which imply the presence of NaCl crystals, as ethanol has a considerable solubility for NaCl.

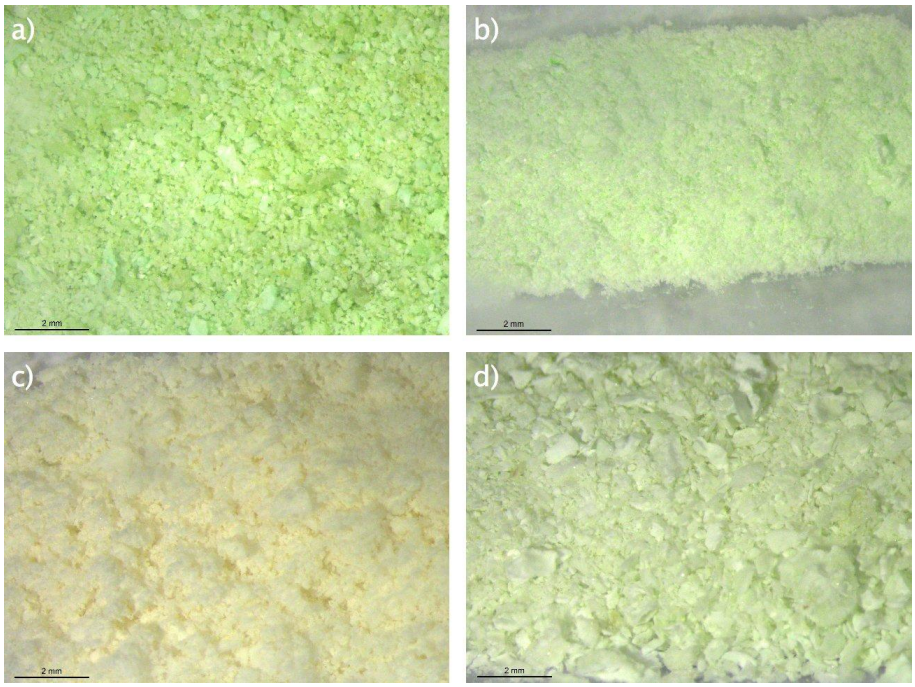


Fig. 3. *Optical micrographs: (a) ESM, (b) ESMc, (c) ESMa and (d) ESME. ESM, ESMc and ESME share a green apparence, while ESMa appears canary. ESMc and ESMa present a finer apparence than ESM. LEICA microscope (M80, obj x1). Scale bar corresponds to 2 mm.*

3.3. Pro-osteogenic effect of ESM relies on the cations

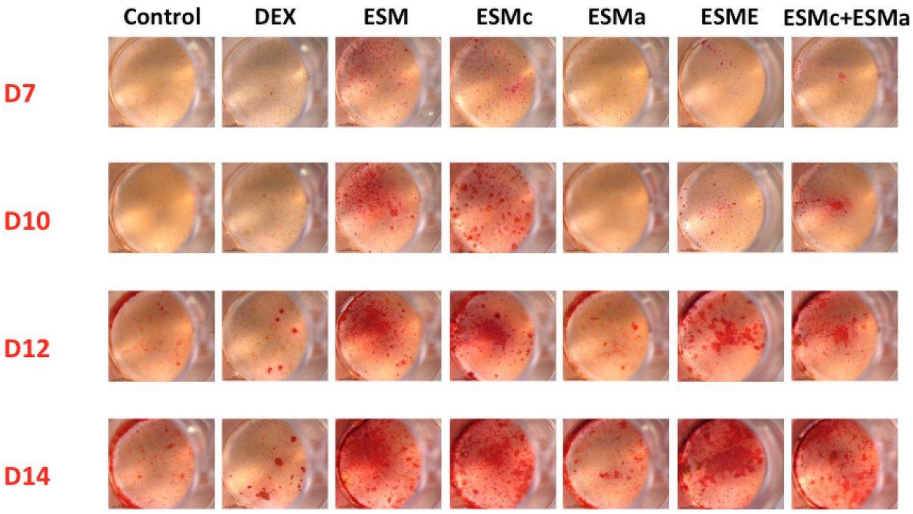
The ionic fractions of ESM are then tested in MC3T3-E1 cells and OA osteoblasts to verify the previous hypothesis. Besides, we wonder if there were an interaction between ESMc and ESMa, thus, an extra condition is added, for which the cells are treated with both ESMc and ESMa at the dose as treated respectively. The results of Alizarin Red staining and Alizarin Red assay are shown in Fig.4 for MC3T3-E1 cells, and in Fig.5 for OA osteoblasts.

3.3.1. Tests on MC3T3-E1 cells

In MC3T3-E1 cells, ESM, ESMc, ESME and ESMc+ESMa induce a cell mineralization from 7 days of treatment, and the mineralization increase with the time of treatment till the end of test. It seems that the mineralization by ESMc+ESMa is not stronger than that by individual ESMc. Dexamethasone has also an osteogenic effect for this cell line, but from 12 days, later than ESM fractions. The

mineralization in control group is visualized from 12 days due to the two osteoinducers, ascorbic acid and β-glycerophosphate, and the mineralization in ESMa group belongs to this type.

a)



b)

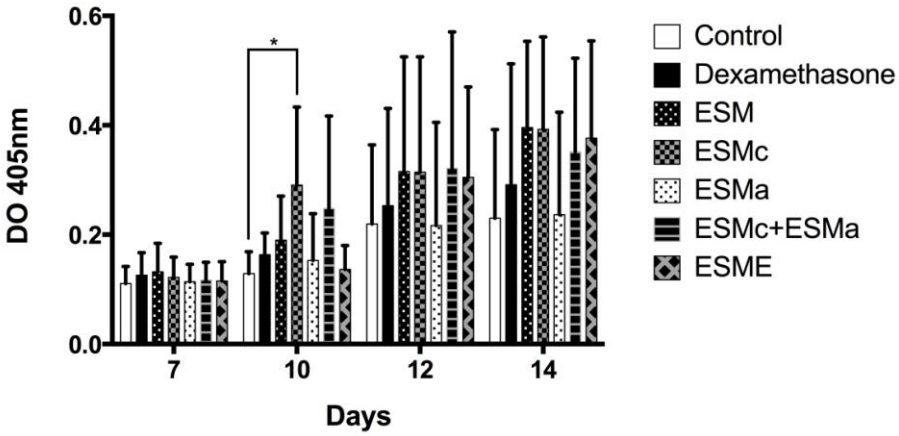
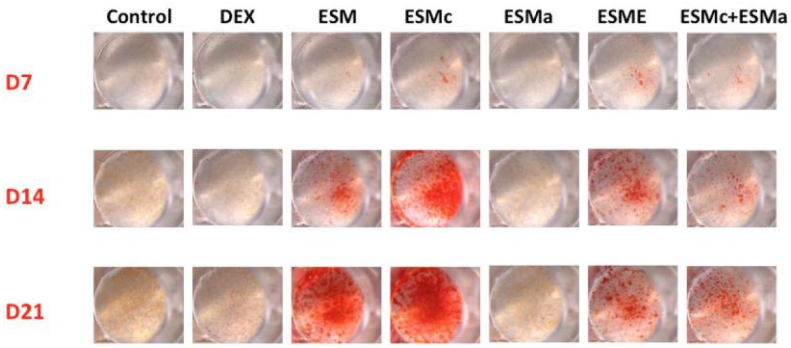


Fig. 4. *ESMc increases mineralization of MC3T3-E1 cells. The cells (a) were treated with ESM (200 µg/ml) and ESM fractions (200 µg/ml) to induce mineralization. Cells not treated or treated with dexamethasone (100 nM) were used as control and positive control respectively. The cells were stained by Alizarin Red after 7, 10, 12 and 14 days of treatment and observed by optical microscopy (LEICA, M80, obj x1). Scale bar is 2mm. Alizarin Red assay (b) measured at 405 nm. t-test: * : P<0.05.*

The different mineralization degrees between the ESM fractions and control are so obvious that we could even determine visually, while, there is a significant difference only between ESMc and control at 10 days (Fig.4b). But it is not hard to notice that the doses of Alizarin Red for each condition correspond very well to the staining in Fig.4a, and all doses from 10 days have a wide standard error of the mean. Thus, the disaccord between the staining and assay is comprehensible. Because, although MC3T3-E1 is a mature cell line, as the passage of cells increases, the cells mineralize faster than those in earlier passage in the presence of osteoinducers.

3.3.2. Tests on OA osteoblasts

a)



b)

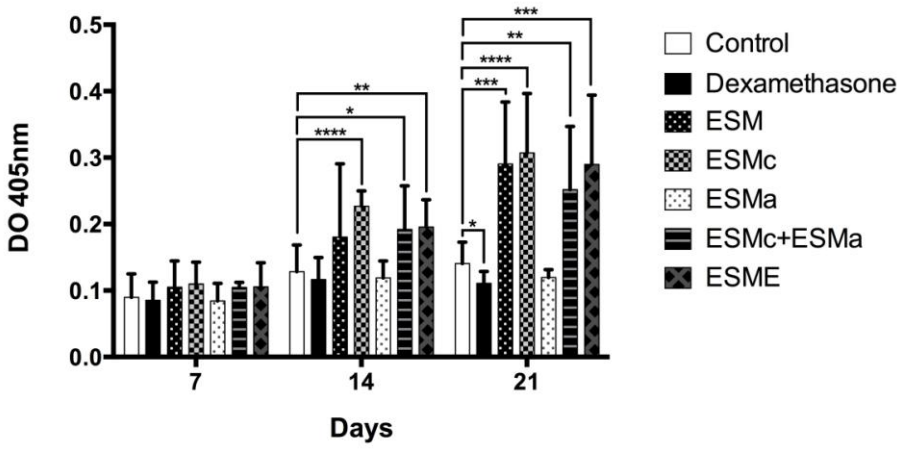


Fig. 5. *ESMc increases mineralization of human OA osteoblasts. The cells (a) were treated with ESM (200 µg/ml) and ESM fractions (200 µg/ml) to induce mineralization. Cells not treated or treated with dexamethasone (100 nM) were used as control and positive control respectively. The cells were stained by Alizarin Red after 7, 14 and 21 days of treatment and observed by optical microscopy (LEICA, M80, obj x1). Scale bar is 2mm. Alizarin Red assay (b) measured at 405 nm. t-test: * : P<0.05; ** : P<0.01; *** : P<0.001; **** : P<0.0001.*

The human OA osteoblasts are tested as the MC3T3-E1 cells, but the staining is performed at 7, 14 and 21 days (Fig. 5a). The staining results are extremely similar to those in MC3T3-E1. ESM, ESMc, ESME and ESMc+ESMa induce a cell mineralization from 7 days of treatment, which increase with the time of treatment till the end of test. No mineralization induced by ESMa is observed. While, dexamethasone has no osteogenic effect for OA osteoblasts, even in our previous study, the mineralization induced by dexamethasone in OA osteoblasts is scarely observed, as in control. This confirms the results observed in the previous study (Brion et al., 2015).

When compared to control (Fig. 5b), ESMc, ESME and ESMc+ESMa have a higher degree of mineralization from 14 days, also for ESM but from 21 days, no significant difference for ESMa, and a lower degree of mineralization for dexamethasone at 21 days. Similarly, the non-significance between ESM and control at 14 days may relate to the wide standard error of the mean of ESM, in combining with the results of other ESM fractions as the significance is found in the later study (Fig. 10b). As for the significance between control and dexamethasone at 21 days, a larger sample number may be needed to validate the effect, as the difference here is rather subtle, and opposite to the later study (Fig. 10b). No significant difference is observed between ESM and ESMc, ESMc and ESMc+ESMa. ESM and ESM fractions have a stronger osteoinduction capacity than dexamethasone, sooner or later during the 21 days of treatment (not shown in figure).

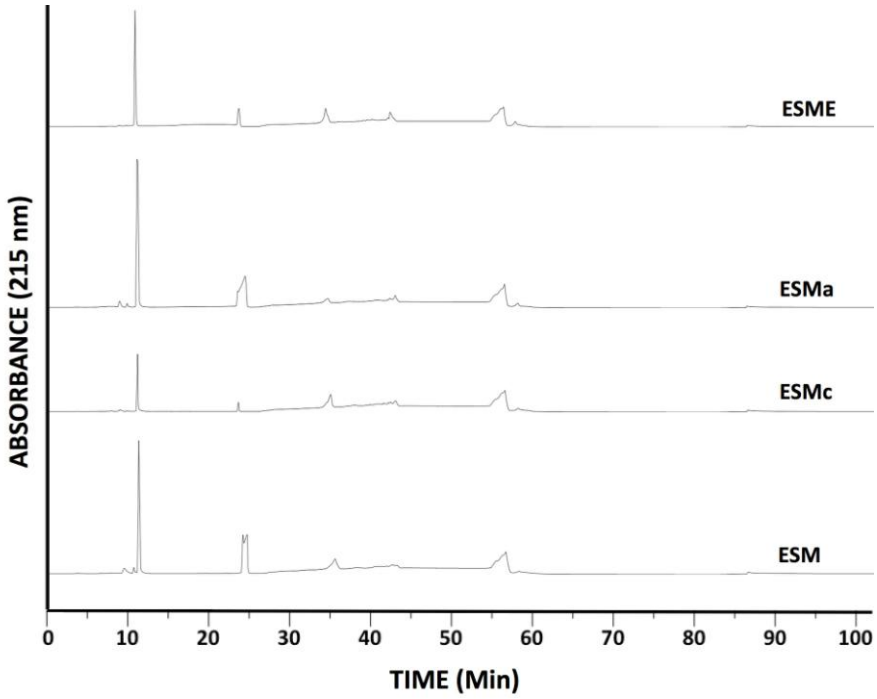
Taking together the results in both cells, we believe that the hypothesis mentioned above is confirmed, the ESMc is responsible for the osteoinduction capacity of ESM, and no interaction with

ESMa is observed. The lost cations are recycled as ESME and demonstrated active. Thus, the cations in ESM need to be investigated further to approach the nacreous osteoinductive compounds.

3.4. Being separated by cation exchange HPLC, ESMc, ESMa and ESME are coherent with ESM

To continue, we try to use HPLC to separate ESM. In consideration of the characters of osteoinductive compounds in ESM, precisely, being organic molecules and positive charge, a cation exchange HPLC chain is chosen. As described in Methods part, based on the ERLIC method (Alpert, 2008), we develop a new program for cation exchange HPLC (Table 1), which permits to separate the solutes in ESM by the difference in polarity and positive charge. The more polar and charged a solute, the later it elutes. As the organic molecules is widely supposed to be proteins or peptides (see Introduction part), the detection is done at 215 nm and at 254 nm for the detection of single bonds for double bonds respectively. Then the graphs of ESM, ESMc, ESMa and ESME are compared respectively at 215 nm (Fig. 6a) and 254 nm (Fig. 6b).

a)



b)

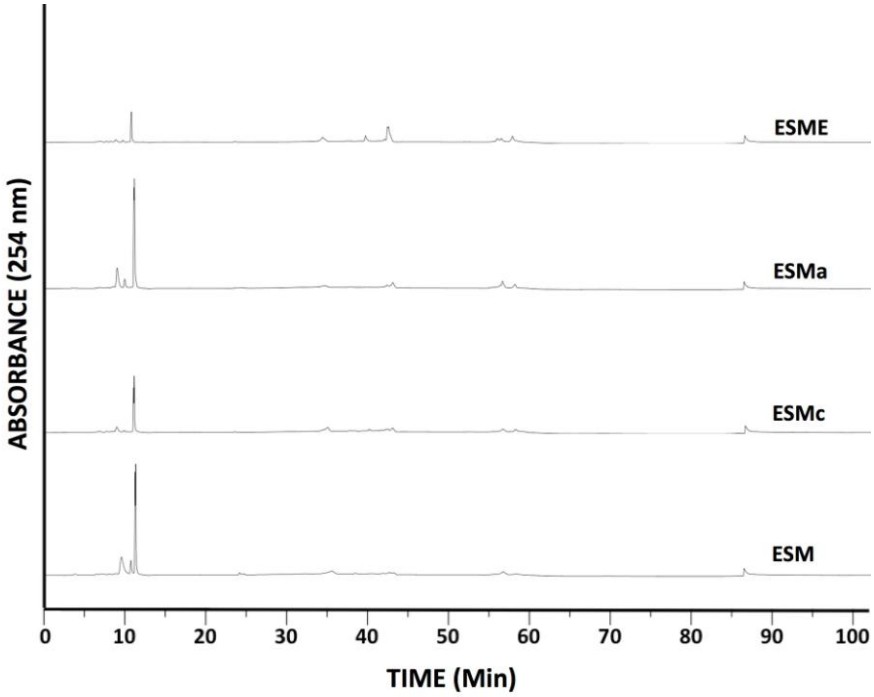


Fig. 6. *Comparison between ESM and ESM fraction by cation exchange HPLC at 215 nm (a) and 254 nm (b). Interchim Column UP5SCX-250/046 is used, in gradient mode, with a personal developed program (see Table 1) to separate the samples. At both wavelengths, the peaks appearing in ESMc, ESMa and ESME are all observed in ESM, but the absorbance intensity of each peak depend on the samples.*

At 215nm (Fig. 6a), 10 peaks are visually separated in ESM, respectively at 9.4, 10.8, 11.3, 23.9, 24.1, 36.1, 43.5, 56.1, 58.0 and 86.6 min of elution. All the peaks observed in ESMc, ESMa and ESME could be found in ESM, suggesting that the ESM fractions are all origin from ESM and no contamination of organic molecules occurs during the multi-procedure extraction. When compared to ESM, the peaks in ESMc and ESMa are complementary, the peaks disappear in ESMc (peak at 10.8 and 24.1 min) can be found in ESMa, and the peak which has a poor absorbance (peak at 36.1 min) has a more intense

absorbance in ESMc, and so as to the peak at 9.4 and 11.3 min but in contrary. In addition, ESME corresponds strictly with ESMc, and present a higher absorbance. While, the last 3 peaks, precisely, the peak at 56.1, 58.0 and 86.6 min, are found also in injection of control (with water, data not shown), thus, the peaks are from the mobile phase, explaining their appearence in all the four samples and the rare difference among them. The graphs at 254nm (Fig. 6b), supports completely the observation and discussion at 215nm, but the absorbance decreases enough that the peak at 23.9 and 24.1 min couldn't be visual. However, in ESME, the peak at 11.3 min is less intense than in ESMc, in contrary, the peak at 43.5 min in ESME is very outstanding in all 4 samples, suggesting that the peak at 43.5 min is more complex in composition than the other compounds, as peptide or protein. Compared to ESM, a new peak appears at 40.0 min in ESMc and the absorbance increases in ESME, while looking back at 254 nm, ESME has a tiny absorbance at 40.0 min, thus, we believe the peak at 40 min is from ESM but not visual due to a low absorbance in ESM.

The results demonstrate that ESME is certainly a mixture of ESMc and NaCl. As ESME is totally from the cation exchange resin, conversely, it proves that ESMc is composed absolutely of a cationic part of ESM, and the anion exchange with resin by using the current protocol is efficiency and credible. Although the absorbance of peak at 11.3 and 24.1 min is also important in ESMa, that's not obliged to conclude that the cation exchange with resin is not credible, as organic molecule, the conditions of charge are sometimes complex and even ambident. Even though, there is still a preference of ionization, acid or basic, for these molecules, that's why the peaks appearing in ESMc and in ESMa are all complementary. For the instance, the ambident ionization doesn't matter the research of active compounds in ESM, as the cation exchange resin has well caught the cationic and active compounds. Anyway, the peak at 23.9, 36.1, 40.0 and 43.5 min deserve more attention for the identification of osteoinductive compounds in ESM, because a relative concentration in ESMc and ESME is observed when compared to ESMa.

3.4. *Collection of ESM on cation exchange HPLC*

As the solvent and buffer solution used in mobile phase are all volatile during a lyophilization, a collection of ESM is performed under the same conditions subsequent to the separation. The schema for collection is presented below (Fig. 7).

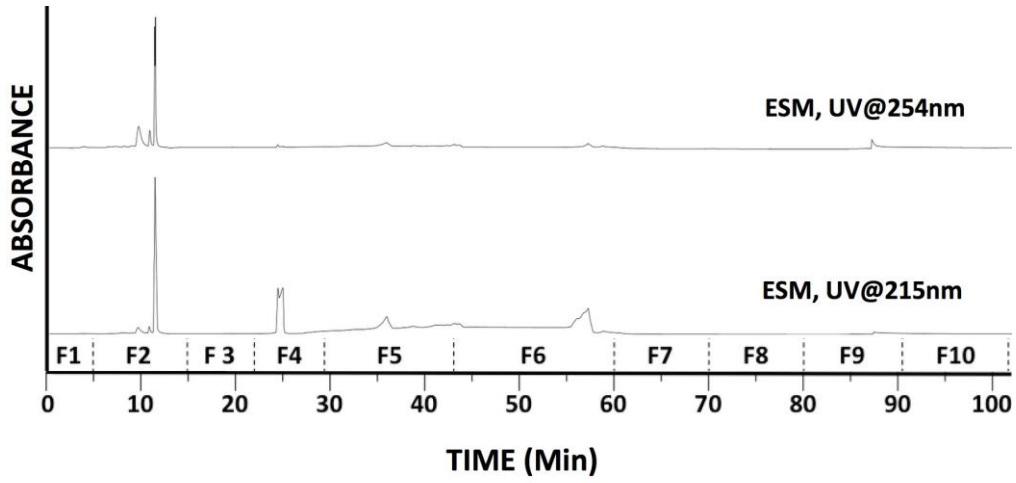


Fig. 7. *10 HPLC fractions of ESM are collected in combining with the absorbance at 254 nm and 215 nm. A cation exchange column (Interchim Column UP5SCX-250/046) is used, in gradient mode, with a developed program (see Table 1) to separate the samples. Note that the collection included the whole elution, and the graphs at 254 nm and 215 nm are not at the same absorbance scale.*

The elution of ESM is fractioned to collect in combining with the absorbance at 254 nm and 215 nm, with the purpose of collecting peak by peak. As the peak at 9.4, 10.8 and 11.3 min, and the peak at 23.9 and 24.1 min are too close, the peaks are collected as a group of peaks. The collection for the peak at 23.9, 36.1, 40.0 and 43.5 min is well considered, initially, they should be collected respectively. But, the peak at 40.0 min is close to both the peak at 36.1 min and at 43.5min, and more than a hundred of injections are predicted for the full collection. To avoid a possible contamination between the fractions, also considering the mobile phase has changed from 40.0 min, the peak is finally collected together with the peak at 36.1 min as F5 (Fraction 5). The peak at 23.9 min and at 43.5 min is collected respectively as F4 and F6. The collection includes the whole elution

to avoid any possible loss of molecules. Finally, 10 fractions are collected, pooled and evaporated respectively before the lyophilization. The evaporation is performed at 40°C, 235 mBar at 100rpm by Rotavapor (Heidolph, Hei-Vap).

The results of collection are listed in Table 2, and some optical micrographs of the fractions *in situ* after the lyophilization are supplied as supplementary data 1. 10 fractions, F1 to F10, most of which have a poor proportion in ESM, only about 1% for each, including F4, while, F5 and F6, take the major proportion, 30.4% and 60.7% respectively. The recovery rate indicates that ESMc may make up the major part of ESM in mass, as F5 and F6 are concentrated in ESMc. While, F7 to F10, collected during the washing period, have also a proportion, confirming that the normal elution time is not enough to elute all the molecules in ESM and a washing program is needed to well condition the column before a new injection. In addition, the collection is demonstrated effective, as the proportion of F7 to F10 are poor, the needed fractions are well collected.

Then the fractions are prepared to test their osteoinductive capacity in cells. But an obvious concern is that, excepting for F5 and F6, the most of the fractions have not an enough quantity for even one test at a classic dose, thus, to avoid the unequal dose between the fractions, also a consideration of possible toxicity, the solution of each fraction is prepared by imitating their dose in ESM. In the future, we will have to develop a more sensitive activity-screening tool to test the compounds present in small amount. A pre-test is firstly conducted in MC3T3-E1 cells. F5 and F6 are observed to have induced a formation of mineralization nodules (data not shown). Afterwards, F5 and F6 are prepared at classic concentration and treated onto the two cell models systematically to validate their osteoinductive capacity (see part 3.6). Before that, some observations of the purified ESM fractions under Scanning Electron Microscopy are performed (see part 3.5).

Table 2

Recovery rate of 10 HPLC fractions of ESM.

Fraction	Time of collection (min)	Mass (mg)	Percentage (%)
F1	0.00-5.00	2.0	0.4
F2	5.02-15.00	6.1	1.1
F3	15.02-22.00	2.5	0.5
F4	22.02-29.00	5.3	1.0
F5	29.02-43.00	169	30.4
F6	43.02-60.00	342.7	61.7
F7	60.02-70.00	8.7	1.6
F8	70.02-80.00	7.0	1.3
F9	80.02-91.00	5.6	1.0
F10	91.02-102.00	6.2	1.1
		555.1 (total)	100 (total)

3.5. A SEM observation into the ESM fractions and elemental analysis by EDS

ESM, and the most important ESM fractions, ESMc, ESMa and F6, are observed under SEM, with the purpose to compare the exterior appearance at micro scale, revealing directly or indirectly some characters of the osteoinductive compounds. Simultaneously, the topical elemental analysis at several positions of the samples is performed by EDS to supply a view on the chemical composition of ESM fractions. The SEM micrographs are presented in Fig. 8. The results of EDS analysis is shown as supplementary data 2.

SEM images (Fig. 8) show that ESM, ESMc and F6 are all a mixture of organic matrix and mineral crystals (Fig. 8a, c, g), but organized differently. In ESM, the crystals are embedded into the organic

matrix, but easily recognized (Fig. 8b), while in ESMc, the crystals are separated with the matrix (Fig. 8d), what's more, in F6, the organic matrix is even loose and fiber-like, and the crystals are very irregular (Fig. 8g, h), suggesting that the crystals are formed in a different solution environment from ESM and ESMc. After all, the micrographs of F6 present an evidence of purification, as the organic part exists in a certain form. The most interesting discovery is that, ESMa is nearly composed of pure and regular crystals, no matrix is observed (Fig. 8e, f).

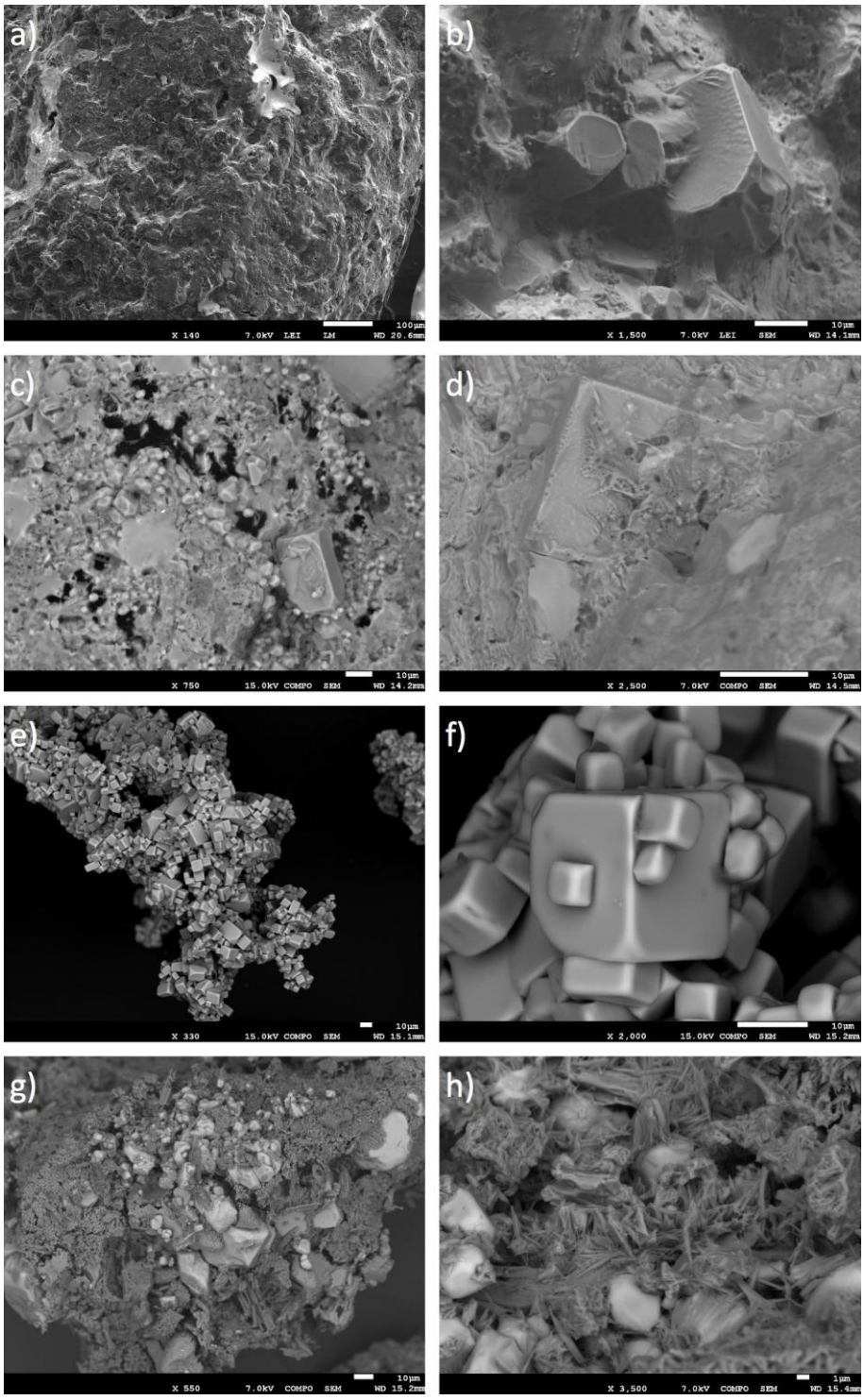


Fig. 8. *SEM micrographs of (ab) ESM, (cd) ESMc, (ef) ESMa and (gh) F6. (a) ESM is a mixture of organic matrix and mineral crystals, (b) and the crystals are embedded into the organic matrix, but easily recognized; (c) ESMc is also a mixture like ESM, but the crystals are separated with the matrix, (d) when focused onto the matrix, the crystals are difficult to recognize; (e,f) ESMa is nearly composed of pure and regular crystals, no matrix is observed; (g,h) F6 is majorly made of organic matrix, loose*

and fiber-like, separated with the very irregular crystals. Photograph conditions: (a) LEI mode for (a,b), COMPO mode for (c to h), 7.0kV for (a,b,d,g,h), 15.0kV for (c,e,f), scale bars correspond to 100µm (a), 10µm (b to g) and 1µm (h) respectively.

Energy Dispersive Spectroscopy (EDS) allows one to identify what those particular elements are and their relative proportions in atomic % or in mass %, which gives a possibility to infer the composition of molecules. The results show that, all the crystals observed in Fig. 8, are of NaCl crystals, as Na and Cl make up the majority of the crystals in mass and with a 1 :1 atomic percentage, suggesting that ESMa is almost composed of NaCl. In addition, the abundant presence of calcium and chloride is observed in ESM, ESMc, F5, and especially in F6. While, it is not obliged to verdict that the two elements formed always $CaCl_2$, as the atomic percentage between them varies a lot, it could be 2:1, also 1:0, or 0:1. But certainly, no calcium is detected in ESMa. Thus, it is necessary to test $CaCl_2$ on the cell models to determine the effect of the sensitive element on cell mineralization (see part 3.6).

The results herein may explain some questions hidden above. As for the proportion of HPLC fractions, F4 to F6, which is concentrated in ESMc and ESME, make up in total 92.1% of ESM, implying that the cationic part of ESM (pure cations) takes certainly more than 46% of mass. As we know here, ESMa is almost composed of NaCl, demonstrating that the anionic part of ESM is almost chloride anion. Surely, the anions in ESMc are all chloride anion, suggesting that ESMc makes directly about 92% of ESM. This is meaningful to explain the results of staining in part 3.3, why there is no significant difference between ESM and ESMc on the osteoinductive capacity; the reason is that, the proportions of osteoinductive compounds in the two active matrices are too close. And, in F5 and F6, there is no crystal being composed of calcium, suggesting that the calcium should be incorporated into organic molecules and the molecules are charged generally positive. In this way, the calcium is eluted, collected and present in the cationic fractions, F5 and F6.

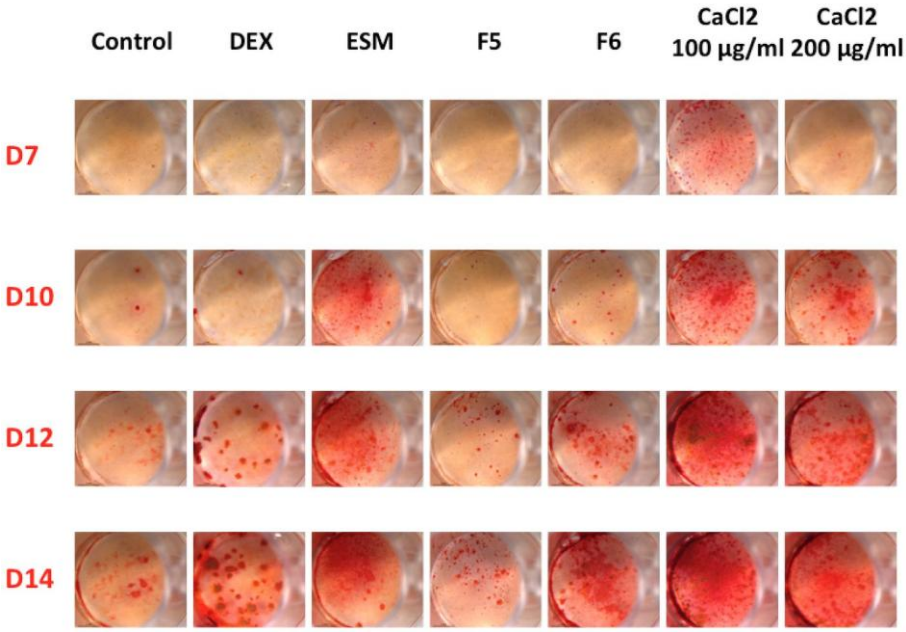
3.6. Pro-osteogenic effet of HPLC fractions of ESM and of free calcium

In this part, we test F5 and F6 on the two cell models to validate their osteoinductive capacity, as discussed in part 3.4. For $CaCl_2$ (VWR. PROLABO. Cat.22328.262), the dose of treatment is yet to decide. The primary concern is the tolerance for the cells. A quantitative analysis of mineral elements on total ESM shows that the calcium takes 16.8% of the mass (data not shown), based on which, we calculate the dose of $CaCl_2$ by imitating the proportion of Ca in ESM; finally, the dose of $CaCl_2$ for the cells is about 100 µg/ml ($CaCl_2$ 100). While, as the dose is not far away from the classic dose 200 µg/ml ($CaCl_2$ 200), we test the two doses on the cells to facilitate some possible comparisons.

3.6.1. Tests on MC3T3-E1 cells

The results are shown in Fig. 9. On the staining (Fig. 9a), an easily visual mineralization is firstly observed in the cells treated by $CaCl_2$ 100 at 7 days, then ESM and $CaCl_2$ 200 at 10 days, dexamethasone and F6 at 12 days, F5 and control at 14 days. After the Alizarin Red assay, generally, the results correspond well to that of staining. When compared to control, $CaCl_2$ 100 from 7 days, ESM from 10 days, $CaCl_2$ 200 from 12 days and dexamethasone from 14 days have a significant difference on the absorbance of Alizarin Red. No significant difference between F5 and control is observed. A significant difference between F6 and control at 7 days is observed, which did not correspond to the staining, because there is a well very intensely but abnormally stained. When compared to ESM, $CaCl_2$ 200 has a significant difference only from 12 days, and $CaCl_2$ 100 at 14 days (not shown in figure). No significant difference between the two doses of $CaCl_2$. To note here, the $CaCl_2$ 200 induces a later mineralization than $CaCl_2$ 100, and both the two conditions have a large standard error of the mean from 10 days when compared to the other conditions.

a)



b)

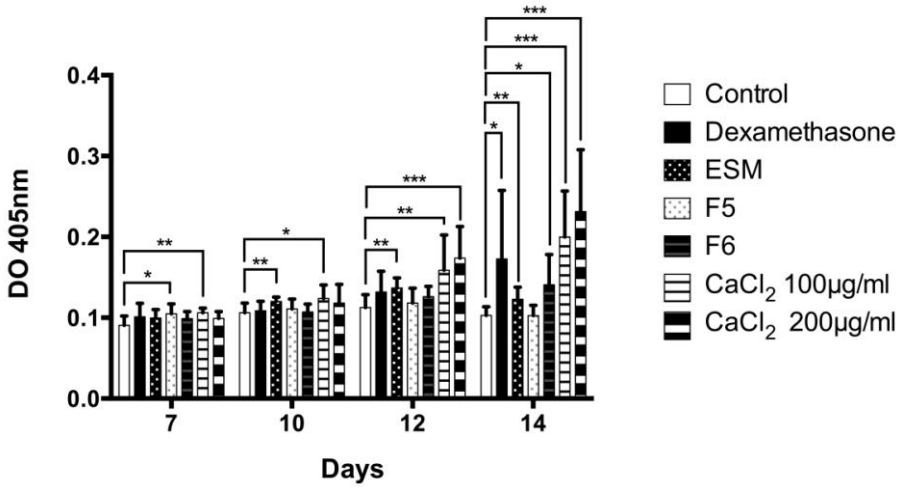


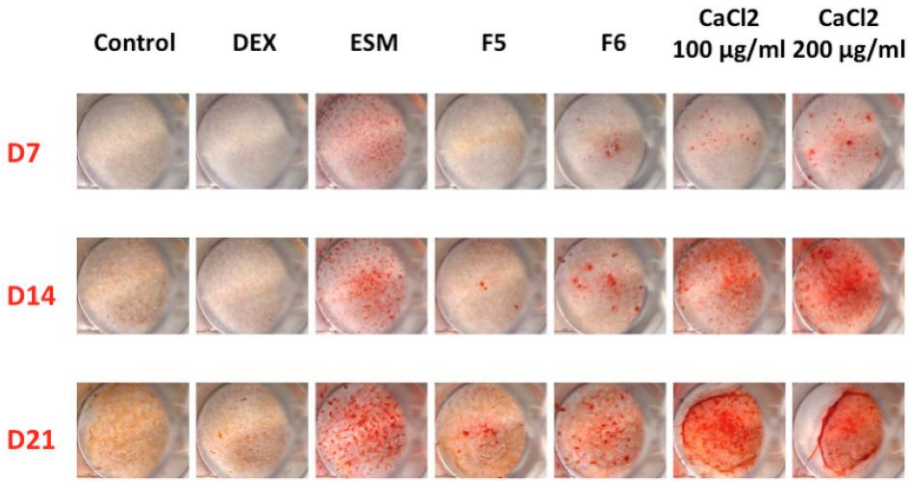
Fig. 9. *F6 and $CaCl_2$ increased mineralization of MC3T3-E1 cells. The cells (a) were treated with HPLC fractions of ESM (200 µg/ml) and $CaCl_2$ (100 µg/ml and 200 µg/ml) to induce mineralization. Cells not treated or treated with dexamethasone (100 nM) were used as control and positive control respectively. The cells were stained by Alizarin Red after 7, 10, 12 and 14 days of treatment and*

*observed by optical microscopy (LEICA, M80, obj x1). Scale bar is 2mm. Alizarin Red assay (b) measured at 405 nm. t-test: * : P<0.05; ** : P<0.01; *** : P<0.001.*

3.6.2. Tests on OA osteoblasts

The results are shown as Fig. 10. In the staining part (Fig. 10a), the mineralization is visually from 7 days by ESM, F6, $CaCl_2$ 100 and $CaCl_2$ 200, from 14 days for F5, no mineralization is observed in the two controls. Note that the mineralized cell layers treated with $CaCl_2$ have a tendency to detach from the dish. As for the assay (Fig. 10b), when compared to control, ESM, $CaCl_2$ 100 and $CaCl_2$ 200 have a significant difference from 14 days, F6 has no significant difference, while, dexamethasone has a significant difference at 14 days, as discussed in part 3.3, it is not credible. In addition, ESM has a significant difference over both dose of $CaCl_2$ at 7 days (not shown in figure). No significant difference between the two doses of $CaCl_2$. Note the large standard error of the mean for $CaCl_2$ 100 and $CaCl_2$ 200 from 14 days.

a)



b)

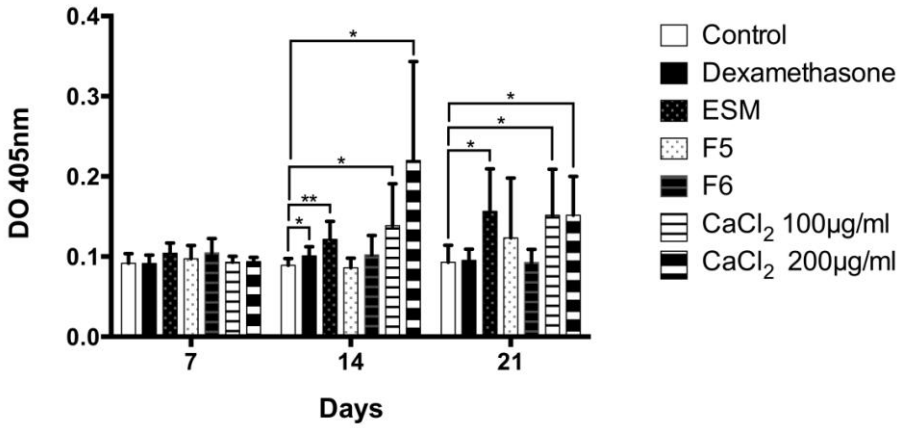


Fig. 10. *$CaCl_2$ increased mineralization of human OA osteoblasts. The cells (a) were treated with HPLC fractions of ESM (200 µg/ml) and $CaCl_2$ (100 µg/ml and 200 µg/ml) to induce mineralization. Cells not treated or treated with dexamethasone (100 nM) were used as control and positive control respectively. The cells were stained by Alizarin Red after 7, 14 and 21 days of treatment and observed by optical microscopy (LEICA, M80, obj x1). Scale bar is 2mm. Alizarin Red assay (b) measured at 405 nm. t-test: * : P<0.05; ** : P<0.01.*

Taking together the tests of mineralization on MC3T3-E1 cells and OA osteoblasts, we have a impression that F5 and F6 have an osteoinductive capacity, but not stable enough, giving scarcely a significant difference for the mineralization compared to control. A further study may be needed to adjust the collection conditions to test the possible interaction between the cationic fractions.

The mineralization and assay related to $CaCl_2$ are outstanding, even induces a stronger mineralization than ESM. While, there is no difference between the two doses, in contrary, at early days of treatment, $CaCl_2$ even inhibits the mineralization to an extent. And the mineralization induced by $CaCl_2$ seems also less stable than ESM, as the absorbance of Alizarin Red is frequently seen at a large standard error of the mean . Also, the tests on OA osteoblasts are initially designed as long as 28 days, but the cells treated with $CaCl_2$ have a obvious tendency to proliferate rather than to differentiate when compared to the other cells, resulting in a severe detachment at 28 days and impossible to perform the assay and comparison. What's more, when looking into the mineralization nodules at a higher magnificence under optical microscopy, we find that the nodules induced by ESM and $CaCl_2$ are similar but not completely the same (shown as supplementary data 3), which needs to be analyzed by using Raman spectroscopy.

(Mahamid et al., 2011) demonstrate that, *in vivo* osteoblasts actively produce disordered mineral packets within intracellular vesicles for mineralization of the extracellular developing bone tissue, and the phosphate entities are first formed and thereafter gradually sequester calcium within the vesicles. This mode of mineral deposition into collagen matrices, is namely that an amorphous mineral precursor is transiently formed and deposited within the gap zones inside the collagen fibril, followed by crystallization into hydroxyapatite (Mahamid et al., 2010)(Olszta et al., 2007)(Weiner, 2008). This strategy is widely employed by many invertebrates for the formation of their calcium carbonate mineralized tissues (Weiner et al., 2009). Our *in vitro* results on $CaCl_2$ confirm this study and an *in vitro* study in osteoblastic ROS17/2.8 cells (Matsuoka et al., 1999), as the free Ca induces cell mineralization by a supply of calcium. But the free Ca is not the best source for bone

mineralization. As revealed by (Hamdi et al., 2015), although $CaCl_2$ has a highest solubility at different pH, but has a lower osteoinduction capactity than limestone and fat-encapsulated tricalcium phosphate in broiler chickens, implying that some other molecules are involved to regulate the process. To date, the regulating molecules are supposed to be proteins or peptides (Rousseau et al., 2008), 2003). Calcium is also proven promoting cell proliferation (Takita et al., 2006), and osteoblastic differentiation (Matsumoto et al., 2013), even at 0.35 mmol/L, corresponding to our observations.

4. Conclusion

We have developed a new method for separating and purifying the nacre extracts by an ionic approach. By this method, we prove that the pro-osteogenic effect of ESM relies on the cationic part, without interaction with the anionic part. The osteoinductive compounds we seek may supply a regulating signal to make the process moderate and correct. The calcium in ESM is demonstrated to play a role in inducing the mineralization, and should be incorporated into organic molecules, precisely, into the cationic parts. Interactions between the cationic fractions are possible.

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Authors' roles: GZ, ASW, AB, MHP, VM, AB, DM, LG, PG and MR designed and performed the experiments. GZ and MR wrote the manuscript. MR accepts responsibility for the integrity of the data analysis.

Conflict of interest

The authors declare that they have no competing interests.

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