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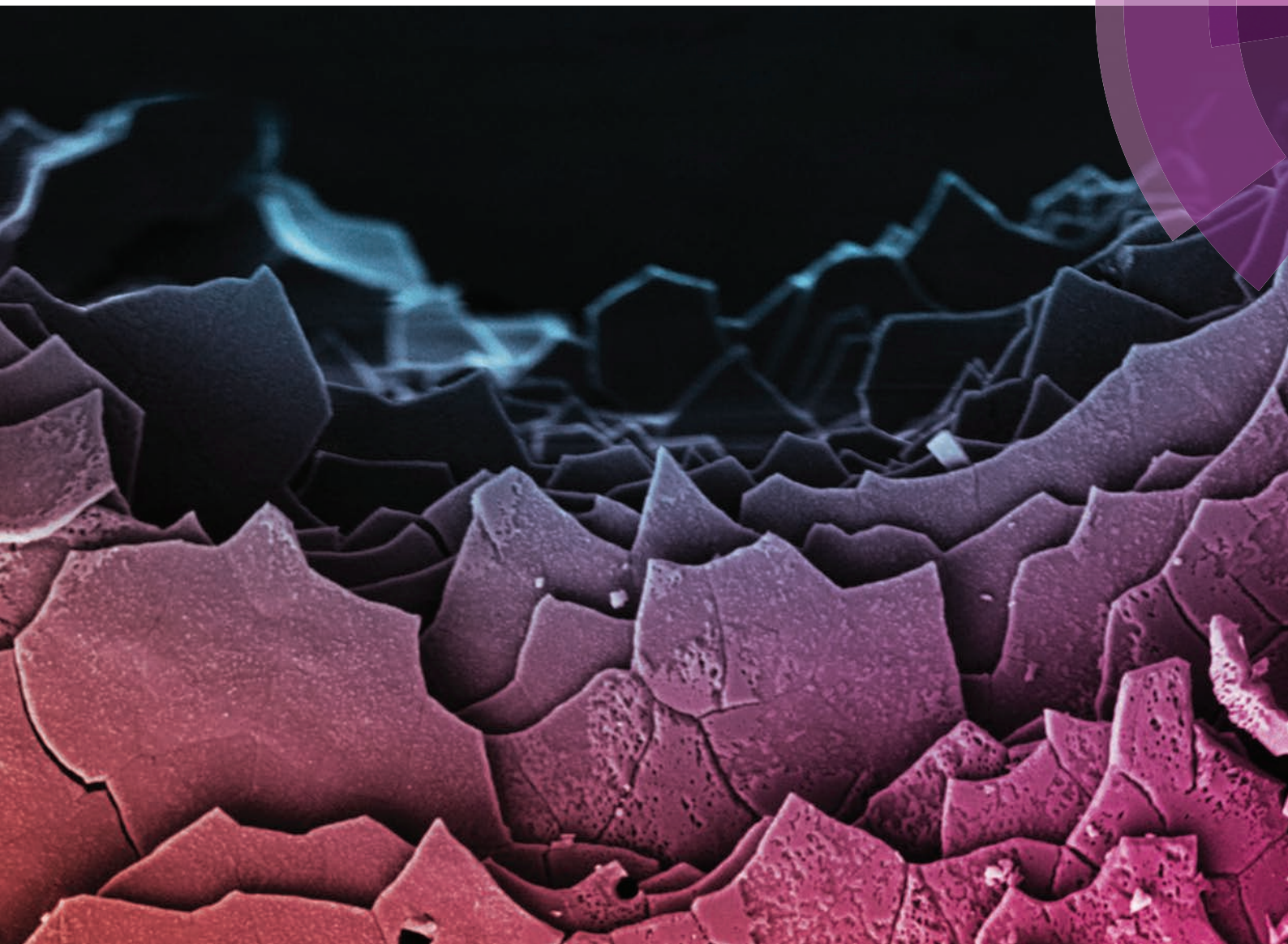
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**COMMUNICATION**
Colin L. Raston *et al.*
Evaluating the effects of nacre on human skin and scar cells
in culture

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# Evaluating the effects of nacre on human skin and scar cells in culture†

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**Pearl nacre, a biomineralisation product of molluscs, has growing applications in cosmetics, as well as dental and bone restoration, yet a systematic evaluation of its biosafety is lacking. Here, we assessed the biocompatibility of nacre with two human primary dermal fibroblast cell cultures and an immortalised epidermal cell line and found no adverse effects.**

There are three main types of pearl oysters of the genus *Pinctada*: the "Akoya" pearl oyster called *Pinctada fucata*, the "Golden lipped" oyster *Pinctada maxima* and the "Black lipped" oyster named *Pinctada margaritifera*. Mollusc shells are mainly made up of two layers of calcium carbonate, comprising an outer layer of calcite and an inner layer of aragonite. Nacre (mother of pearl) in all oyster shells is a calcified structure that forms the lustrous inner layer. It is mainly composed of aragonite (~95–97%) tablets oriented in multiple layers, each surrounded by organic matrix.[1,2] This organic matrix makes up ~5% of the nacre composition and is mainly comprised of polysaccharides and proteins.[3] According to a European Commission report published in 2007 the cosmetic and toiletries industry in the EU, Japan, China and the US had a total market value of €136.2 billion.[4] The cosmetics industry maintains its edge by constantly developing novel topical skin treatments. A popular example is the use of all-natural or organic ingredients, such as fruit and plant extracts to offer wrinkle relief that mimics the painful and potentially dangerous side effects associated with invasive chemical remedies.[5] Clinically, topical treatments containing, for example, aloe vera, vitamin C, corticosteroids and tacrolimus are used with

the aim of minimizing scarring.[6] Recently, there has been interest in the cosmetics industry in the use of nacre as a key ingredient.[7] Most of the formulations are reported to either use powdered pearl shell or powdered nacreous layer shell. Powdered shell and powdered nacre comprises of both organic and inorganic components. It is reported that nacre stores in its mineral-based organic structure a variety of bioactive molecules. Efficacy of this water soluble matrix (WSM) has been tested in a porcine burn injury model.[8] WSM was obtained by suspending powdered nacre in ultra-pure water and collecting the supernatant *via* precipitation of insoluble components by centrifugation. It was concluded that the active mineral based organic component has beneficial effects on the skin with enhanced wound healing.[8,9]

Nacre has also attracted attention for its potential in supporting bone grafting and bone regeneration. In culture under physiological conditions, nacre can transform to hydroxyapatite, the phosphorous rich main constituent of the mammalian bone framework.[10,11] Nacre and its WSM can also aid in osteogenic regeneration.[9,12–17] High phosphorous rich domains have been described at the interface between bone and implants made from *Margaritifera* shells which are biocompatible, biodegradable and osteoconductive and thus are thought to promote bone formation.[18] Furthermore, nacre powder has been used as an implantable material for reconstruction and regeneration of maxillary alveolar ridge bone in humans.[19] In this example, the implanted nacre dissolves gradually and is eventually replaced by the mature lamellar bone suggesting that the nacre acts as a biocompatible substrate for bone replacement.[19] The water soluble components of the crushed nacre have also been investigated for their potential in bone regeneration in a similar vein.[20,21] Lee *et al.*, demonstrated the wound healing potential of WSM component in a deep burn porcine skin model and showed enhanced collagen secretion and deposition at the injury site resulting in enhanced healing.[8] In another *in vivo* study using a rat skin incisional injury model, powdered nacre was implanted between the epidermis and dermis at the incisional site, with an aim of studying the effect of nacre on the synthesis of certain constituents of the dermal

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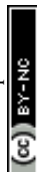
extracellular matrix. It was concluded that implanted nacre increased collagen synthesis by dermal fibroblasts.[22] While extensive investigations have been carried out in bone, the evaluation of the biocompatibility of nacre with human skin cells is lacking. Thus the growing number of cosmetic formulations in the market with nacre as a key ingredient[7,23,24] clearly warrants a thorough assessment with human skin cells. Since scars are also common, and contain cells with a phenotype distinct from normal skin,[25] it is also important to test potential cosmetic ingredients with both cell types.

In the present study, we use nacre from the inner calcified layer of the shell of *Pinctada margaritifera* and report the *in vitro* toxicity assessment of the material on three cell types representing both epidermal and dermal layers of human skin. These were HaCaT cells, a human derived immortalised keratinocytes cell line, primary human dermal skin fibroblasts (HDF) and primary human scar fibroblasts (HSF).

Nacre used in the study was gently scraped[26,27] from the inner layer of the shell to avoid the post processing required in the case of powdered shell. SEM images (Fig. 1) confirmed that the nacre was composed of pseudo-hexagonal shaped aragonite tablets which have basal plane dimensions of 2–6 μm, and a thickness of 300–400 nm.[28] This structure is characteristic of previously reported nacre, which is a composite material consisting of alternating layers of mineral tablets separated by thin layers of biomacromolecular "glue".[29,30]

To test the cytotoxicity of nacre, a live/dead assay was carried out (see ESI S1.4†). Cells were incubated with nacre in culture media at physiological conditions for 24 h to 72 h and were then stained for viability using calcein AM/ethidium bromide I solutions. Viable cells fluoresce green through the reaction of calcein AM with intracellular esterase, whereas non-viable cells fluoresce red due to the diffusion of ethidium homodimer across damaged cell membranes and binding with nucleic acids.

Fig. 2 shows live cells as the percentage of the total cells in human primary dermal skin fibroblast (HDF), human primary scar fibroblast (HSF) and human derived immortalised HaCaT cell cultures when exposed to various concentrations of nacre for 24 h and 72 h. Cytotoxicity of nacre was not observed for any of the concentrations examined in HDF cells (Fig. 2a). However, interestingly at a concentration of 2.5 mg ml$^{-1}$ of nacre (highest concentration tested) there was a significant reduction in viability at both 24 and 72 hours in the HSF cells (Fig. 2b). This underlines the importance of testing both scar and normal skin cell types for cosmetic application. Toxicity was also observed at a concentration of 0.5 mg ml$^{-1}$ in HaCaT cells (Fig. 2c), although this was only observed at 24 hours and
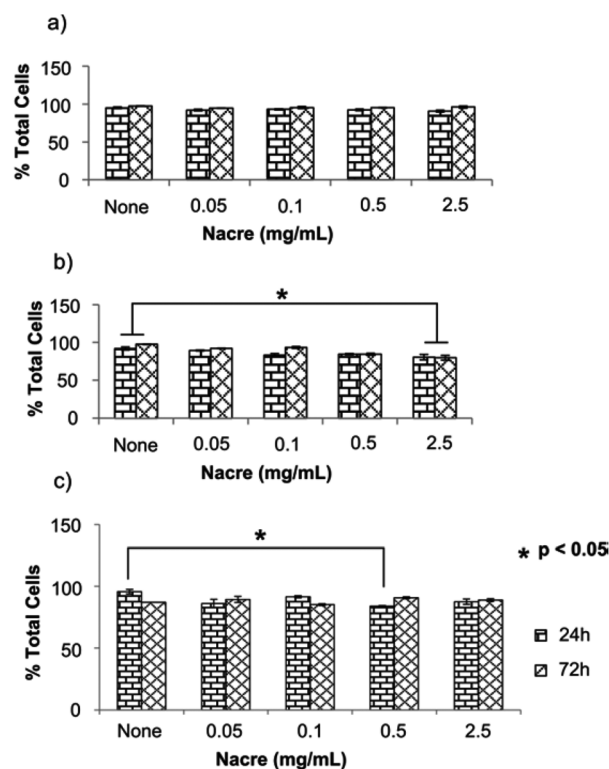


**Fig. 2** Cell viability assays showing percentage of live cells in the culture post incubation with nacre. (a) Human dermal skin fibroblasts cells, (b) human scar fibroblasts cells and (c) human derived immortalised HaCaT cells were incubated with various concentrations of scrapped nacre and treated with calcein AM/ethidium bromide I to stain for live and dead cells. Both live and dead cells were counted using fluorescence microscopy. 'None' is the untreated control. Data presented as average ± SEM (*n* = 4). Significance was set at *$p$ < 0.05 using bonferroni test in one way ANNOVA.
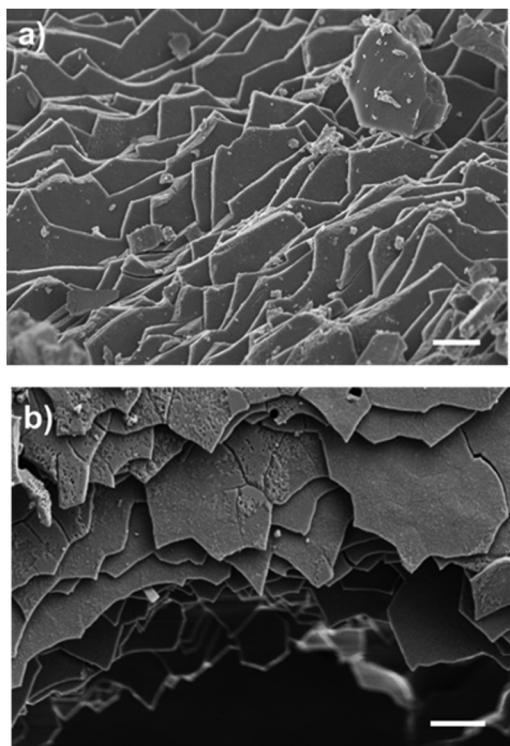


**Fig. 1** Top view of the scrapped nacre, imaged using scanning electron microscopy (SEM). Scale bar (a) 1 μM and (b) 2 μM respectively. Sample was coated with 4 nm platinum prior to imaging.

was no longer present at the 72 hour time point. These results are in line with the results obtained previously where constituents of nacre were shown to promote wound healing in a rat model[22] and deep burn porcine skin.[8] In both these studies, nacre has been shown to promote the recruitment of fibroblasts for restoration and coverage of the injury site while showing no apparent signs of cytotoxicity. It has also been shown to promote bone formation when implanted in the femur of sheep with midshaft hemidiaphysis resection of their femur *in vivo* reiterating the non-cytotoxic advantage of nacre.[31]

Fibroblasts have been reported to undergo morphological changes from dendritic to stellate shapes upon exposure to external cues caused by changes in actin polarisation and adhesion.[32,33] Cell morphology in fibroblasts is known to be influenced by cytokines such as transforming growth factor $\beta$ which can potentially induce polymerisation of globular to filamentous actin.[34] Fibroblast morphology can also be modulated by extracellular matrix architecture during wound healing *via* cell–matrix interaction.[32] Such morphological change has been observed in cells undergoing oxidative stress.[35,36] In our study, we found similar changes in fibroblast morphology for both HDF and HSF cells at the highest concentration of nacre of 2.5 mg mL$^{-1}$ (Fig. 3). Similar altered mor-

phology was also observed for HaCaT cells (see ESI Fig. S1†). It could be postulated that the high concentration of nacre induces cellular stress, resulting in changes in the actin cytoskeleton and a more stellate morphology (Fig. 3i, iii, ii and iv). Cell area was calculated from the fluorescent images shown in Fig. 3 using Image J software.[37] It was found that both HDF and HSF had significantly larger cell areas ($p < 0.05$) when treated with 2.5 mg mL$^{-1}$ of nacre (HDF: 2.79 ± 0.13 μm and HSF: 3.0 ± 0.19 μm respectively) as compared to the non-treated controls (HDF: 1.56 ± 0.08 μm and HSF: 1.54 ± 0.10 μm respectively) (see ESI Fig. S2†).

Altered fibroblast morphology has been thought to occur in response to various factors including aging,[38] strength of the extracellular matrix[39] or other etiologies that induce mechanical stress on the cell. Changes in morphology also commonly indicate oxidative as well as mechanical stress.[39] Therefore, we explored whether the morphological changes and increase in
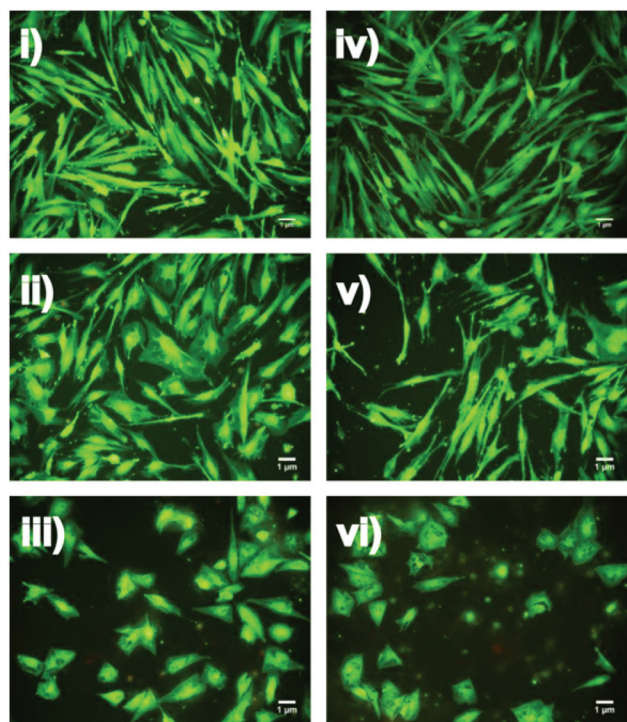


Fig. 3 Cell morphology post calcein AM staining and imaged using fluorescent microscopy. Cells were treated with various concentrations of nacre for 24 h, stained and imaged. (i) untreated (control) primary human dermal skin fibroblasts (HDF), (ii) HDF treated with 0.05 mg mL$^{-1}$ nacre, (iii) HDF treated with 2.5 mg mL$^{-1}$ nacre, and (iv), untreated (control) primary human dermal scar fibroblasts (HSF), (v) HSF treated with 0.05 mg mL$^{-1}$ nacre and (vi) HSF treated with 2.5 mg mL$^{-1}$ nacre. Scale bar 1 μm.
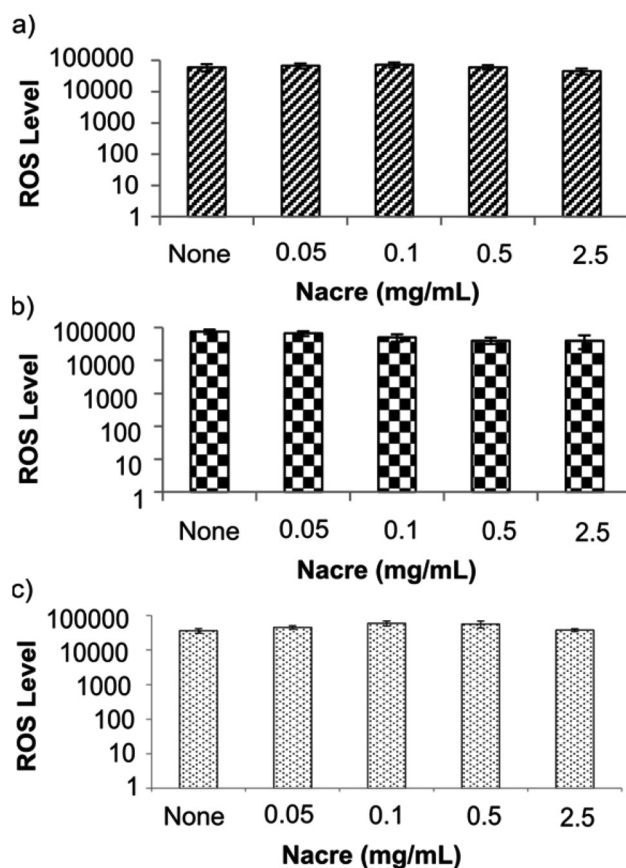


Fig. 4 Reactive oxygen species (ROS) assay showing ROS levels in cells stressed with various concentrations of nacre for 24 h. No significant stress was observed as a result of calcium (from nacre) induced oxidative stress at the concentrations studied. (a) Human dermal skin fibroblasts cells, (b) human scar fibroblasts cells and (c) human derived immortalised HaCaT cells were incubated with various concentrations of scrapped nacre for the specified period of time. Cells were then incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) solution which fluoresce in the presence of reactive oxygen species. 'None' is the untreated control. Data presented as average ± SEM ($n = 3$). Significance was set at *$p < 0.05$ using bonferroni test in one way ANNOVA.

cell area at the highest concentration of nacre in culture was a result of, or induced oxidative stress in, the cells. Oxidative stress was tested using the cell permeable fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is taken up by cells and is deacetylated by cellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH) which is rapidly oxidised by reactive oxygen species (ROS) to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). The fluorescent intensity is proportional to the ROS levels within the cytosol (see ESI S1.5†). Cell responsiveness to the assay was carried out by stressing the cells with the $H_2O_2$ solution provided in the kit which was also used to generate the calibration curve (see ESI Fig. S3†). No changes in levels of reactive oxygen species were observed in any cell type at any concentration of nacre (Fig. 4). This is important as oxidative stress is known to be a significant contributor to skin damage and excessive scarring in previous studies.[40] It has been known that cells alter their morphology depending on their environment.[41,42] It is therefore hypothesized that the altered fibroblast morphology in the present case is mainly due to the regulation of cell motility through geometrical constraint in the presence of nacre. Indeed, it has been previously reported that when cells probe their physical surroundings, they acquire mechanical information or signals that help to determine the direction of migration, with a consequential change in cell morphology.[43]

## Conclusions

We have established the biocompatibility of nacre using three human cell types representing the two primary layers of human skin, using immortalised keratinocytes from the epidermal layer and two primary human dermal cell cultures. The nacre used in the present study showed limited cytotoxicity at high concentrations in scar derived cells, with the morphology of the cells significantly changed by exposure at such concentrations of nacre. No apparent oxidative stress was evident in any of the cell types. Overall, the data support the use of low concentrations of nacre in aesthetic formulations, with the potential for high concentrations to cause changes in skin and/or scar cells which may have impact on efficacy.

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