

# MICROFLUIDICS IN BIOMINERALIZATION AND BIOMIMICKING SYNTHESIS

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## ABSTRACT

Biomaterials have attracted investigations for centuries due to their enormous diversity and extraordinary functionality, which are unmatched by man-made materials. Using microfluidics, we have provided direct experimental evidence that an extrapallial (EP) 28kDa protein can influence the morphology, structure and polymorph that is laid down in the shell ultrastructure. Crucially, this influence is predominantly dependent on the existence of an EP protein concentration gradient and its consecutive interaction with  $\text{Ca}^{2+}$  ions. Furthermore, the functional similarity was found using an engineered counterpart protein (i.e. cloned and expressed), demonstrating a new biomimetic strategy to develop functional biomaterials.

**KEYWORDS:** Biomineralization, biomimetic, microfluidic, EP protein, synthesis

## INTRODUCTION

Biomineralisation is the process in which biological systems exercise strict control over the formation of biominerals such as shell, teeth and bones. An obvious example is seen clearly in the shell of the mollusc *Mytilus edulis* (Fig. 1), where calcium carbonate,  $\text{CaCO}_3$  is deposited as calcite and aragonite, showing well-defined polymorph control. Currently, there is substantial momentum to explore the fundamental mechanisms underlying these biomineralisation processes [1]. An understanding of these processes will lead to new developments in advanced materials and medicines, which may have wide-ranging significance in health care.

Recently, many observations have suggested that the extrapallial (EP) fluid occupying the cavity between the shell and mantle tissue of the mollusc might play an important role in control of shell formation *in vivo* [2-3]. This EP fluid contains proteins, glycoproteins, carbohydrates, and is supersaturated with respect to the shell minerals. Although the major EP fluid protein components of the mollusc *Mytilus edulis* have been isolated and characterized [2-3], their direct role in mollusc shell formation remains undefined. This is mainly due to the fact that complex interactions of ions and biomolecules occur at precisely controlled hierarchical levels that are difficult to replicate using methods based on traditional wet bench procedures [4]. In addition, the identification of specific factors requires vast amounts of testing to screen the many permutations of reactants.

Using microfluidics we have demonstrated the unique advantages of integration of on chip screening with in situ Raman to study polymorph formation in real time [5]. In this work, we investigate the dynamic influence of the most abundant EP protein (both isolated and cloned) on the structure and polymorph of  $\text{CaCO}_3$  crystals, and reveal information that is impossible to obtain using traditional bulk methods.

## EXPERIMENTAL

Extrapallial (EP) fluid proteins were extracted by syringe from the space between the mussel shell and the mantle tissue before being purified using ion exchange and gel filtration chromatography. The most abundant EP protein in *M. edulis* is a 28kDa protein that is highly glycosylated [2-3]. Based on its primary structure [2], its engineered counterpart was cloned and expressed in *E. coli*. Bovine serum albumin (BSA) (Sigma Aldrich) was used as non-functional protein control.

50 mM calcium chloride ( $\text{CaCl}_2$ ) and sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in 100mM MOPS buffer at pH 7.5 were used as  $\text{CaCO}_3$  forming solutions. Protein was added to either the  $\text{CaCl}_2$ ,  $\text{Na}_2\text{CO}_3$  or both solutions at the concentration of 50  $\mu\text{g}/\text{ml}$  prior to on-chip experiments.

T-Junction microfluidic chips that could be reversibly sealed, as described previously, were used in this study [5]. The total length of the serpentine microchannel was 5 cm, with all microchannels having a uniform cross section of 130  $\mu\text{m}$  x 100  $\mu\text{m}$ . Small amounts (5  $\mu\text{l}$ ) of the  $\text{CaCl}_2$  and  $\text{Na}_2\text{CO}_3$  solutions (described above) were delivered using carrier

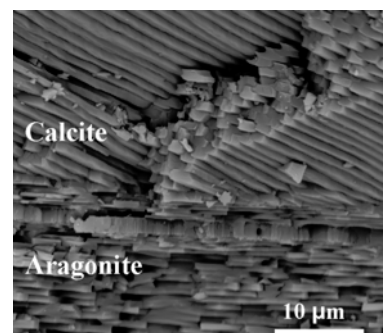


Figure 1: Scanning electron image of a fracture section of the shell of *Mytilus edulis* with calcite prisms to the exterior (top) and aragonite nacre to the interior (bottom).

liquids at the same flow rate of 2  $\mu\text{L}/\text{min}$  (i.e. a velocity of 5.12 mm/s along the channel) through two separated inlets. Optical microscopy was used to record crystal formation and Raman microscopy for identifying the crystal polymorph [5]. Immediately after the experiments, the reversible sealed chip was immersed in pure ethanol to separate the PDMS chip from the glass substrate. The PDMS chip was then dried with nitrogen for subsequent imaging. No changes in crystal morphology and polymorph have been found to result from this treatment (at the optical resolution limit).

## RESULTS AND DISCUSSION

The Reynolds number at the chosen delivery velocity is less than 1, suggesting a laminar flow system. Under this condition, a stable interface forms when the two streams of solutions meet in the T-junction. At this point, due to the limited time for diffusion, a step change in the concentration profiles cross the interface occurs for the species in either sides of the streams. Using computational fluid dynamics software (Comsol), a complete set of concentration profiles for  $\text{CO}_3^{2-}$ ,  $\text{Ca}^{2+}$ , and proteins in the buffered system were constructed. This allows to estimate supersaturation ratio ( $S$ ) of  $\text{Ca}^{2+}$  and  $\text{CO}_3^{2-}$  ions [5]. Supersaturation ratio is a measure of the driving force of the crystallization of the system and thus an indicator of crystal forming locations. Figure 2A shows a representative profile of  $\text{Ca}^{2+}$  at the T-junction (Note: for the clarity, the profile of  $\text{CO}_3^{2-}$  is not shown) and the corresponding supersaturation ratio profile (Figure 2B). The sharp saturation ratio peak in the centre of the channel is coincident with the single-line crystal formation at the interface as seen in Figure 2B.

Using the microfluidic platform and by presenting the proteins either in the  $\text{Na}_2\text{CO}_3$  solution only, in the  $\text{CaCl}_2$  solution only, or in both solutions, we have investigated the influence of both the existence of, and concentration profiles of EP proteins on  $\text{CaCO}_3$  crystallization. Figure 3 shows that the wild type 28kDa EP protein regulates the morphology, structure and polymorph outcome of the crystals at both a micro- and nanoscopic level. Crucially, this regulatory role is predominantly dependent on the existence of an EP protein concentration gradient and its consequent interaction with  $\text{Ca}^{2+}$  ions (Figure 3A&B). Oval-shaped vaterite crystals were formed when the wild type 28kDa EP protein was added to the  $\text{CaCl}_2$  solution only whereas layered calcite crystals formed when the protein was added either to the  $\text{Na}_2\text{CO}_3$  solution alone or to both solutions. In contrast, addition of the non-functional protein, BSA resulted in both spherical vaterite and rhombohedral calcite crystals, no matter which solution component it was added to (Figure 3C).

The observed role of the wild type of 28kDa EP protein in control of crystal formation could not have been observed using conventional bulk methods where rapid mixing is driven by a turbulent flow. As shown in Figure 3D, only single rhombohedral and aggregated calcite crystals formed with no evidence of vaterite crystals.

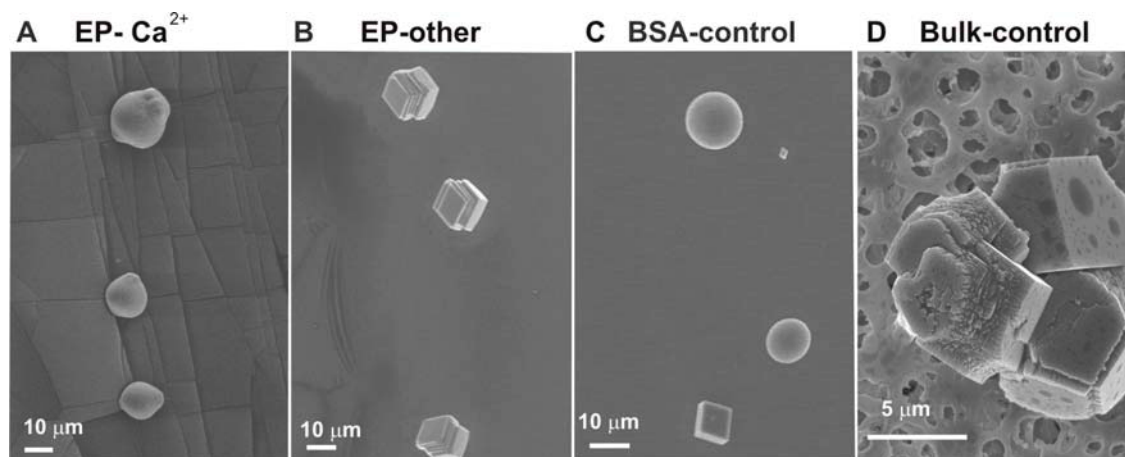


Figure 3. Representative SEM image of crystals formed inside a microfluidic channel at the T-junction (within 100  $\mu\text{m}$ ) (A) when the wild type 28kDa EP protein was present in the  $\text{CaCl}_2$  solution only; (B) in either  $\text{Na}_2\text{CO}_3$  solution only or in both the  $\text{Na}_2\text{CO}_3$  and  $\text{CaCl}_2$  solutions; (C) under negative control when the non functional protein BSA was present; and (D) in a bulk system.

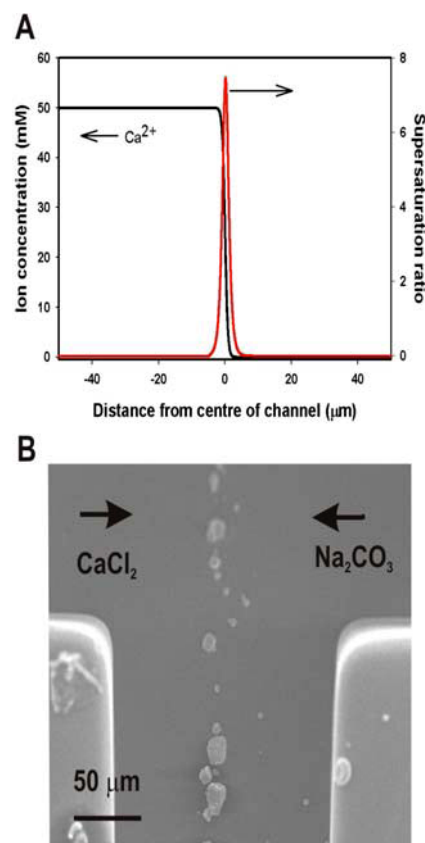


Figure 2. (A)  $\text{Ca}^{2+}$  concentration profile (black) and supersaturation ratio (red) across the channel at the T-junction. (B) A representative image of  $\text{CaCO}_3$  crystals formed at the interface of the two streams.

Importantly, similar phenomena have been found when the expressed counterpart of the wild type 28kDa EP protein was used (Figure 4). Oval-shaped vaterite crystals formed when the expressed 28kDa EP protein was presented in the  $\text{CaCl}_2$  solution only (Figure 4A). It should be noted that the expressed EP protein in this study is not glycosylated, meaning it doesn't contain the carbohydrate moieties that are present in the wild type. This might account for the differences shown in the high resolution SEM images of the surface of the two types of oval-shaped vaterite crystals formed using either the expressed or the wild type EP proteins (Figure 4A II & B). Nevertheless, these findings demonstrate the possibilities of using engineered EP proteins to control crystal formation that could be potentially applied for industrial applications.

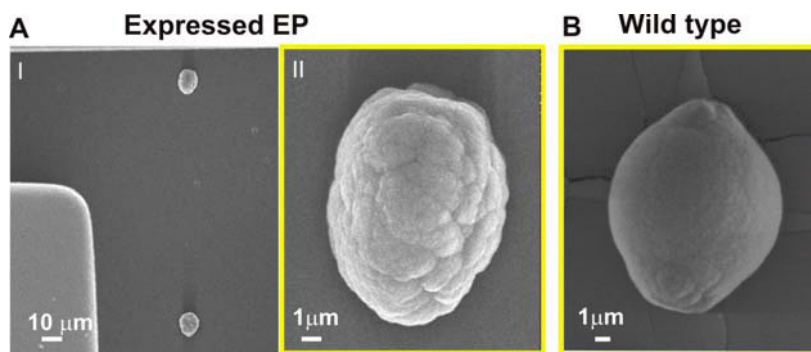


Figure 4. (A) SEM images of the crystals formed in microfluidic channels with the expressed 28 kDa EP protein present in the  $\text{CaCl}_2$  solution only (I) overview in the channel and (II) high resolution of one crystal. (B) High resolution of the oval crystal formed using the wild type of EP protein as shown in Figure 3A.

## CONCLUSION

Using microfluidics, we have shown that that proteins located in the EP fluid adjacent to the shell, can regulate, at both micro- and nanoscopic levels, crystal morphology, structure and polymorph. Most importantly, this regulatory influence is predominantly dependent on the existence of an EP protein concentration gradient and its interaction with  $\text{Ca}^{2+}$  ions. Importantly, the discovery of these findings would not be possible using conventional bulk mixing methods.

Usefully, a functional similarity was found using the expressed analogue of the 28kDa EP protein. This not only provides additional evidence of the key role of this EP protein in the biomineralization process, but also illustrates the potential to scale up synthesis of functional materials via biomimetic strategies.

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