Proceedings of the 9ICEG
9th International Congress on Environmental Geotechnics
25-28 June, 2023 | Chania, Greece
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https://doi.org/10.53243/ICEG2023-447

Synthesis, characterisation, and utilisation of copper nanoflower for biocementation for ground improvement applications

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ABSTRACT

Microbially-induced calcium carbonate precipitation (MICP) has recently emerged as a sustainable ground improvement method. Nevertheless, the technique's applicability in soils with narrow pore throats has been queried. To overcome these challenges, the use of enzymes (including bacterially produced enzymes) was proposed for these soils. However, the use of free enzymes entails many challenges linked predominantly to the limited enzyme supply, the poor stability of the enzyme once released into the soil, and the poor reusability of the enzyme. This paper studies the use of nano enzymes with a high biocementation efficacy for carbonic anhydrase (CA) enzyme delivery as one possible way to overcome potentially these challenges. CA enzyme was used because it has the potential to be an environmentally sustainable biocementation pathway due to its ability to sequester CO_2 for biocement production. The paper presents the synthesis, characterisation, and utilisation of CA-enwrapped copper phosphate-based inorganic hybrid nanoflowers for innovative delivery and enzyme stabilisation due to the enhanced thermal and enzyme activity efficiency and due to their reusability, if recovered at the end of the treatment. The results from this study show that the bovine carbonic anhydrase enzyme enhanced the CO_2 hydration reaction, resulting in a bioprecipitation reaction and the production of calcium carbonate and increased the unconfined compressive strength of treated fine-grained soil with 500kPa for free CA and approximately 1000kPa for the hybrid CA-Cu. The material analysis confirmed calcite as the primary precipitate formed, which would act as a bonding agent between soil particles for ground improvement applications.

Keywords: Carbonic anhydrase, nanoflower carriers, ground improvement, biocementation, CO_2 capture.

1 INTRODUCTION

Soil biocementation via the action of bacteria is emerging as a potentially sustainable ground improvement method, with several claimed advantages compared to conventional chemical ground improvement techniques. However, the technique's applicability in soils with narrow pore throats has been queried. Bacterially produced enzymes rather than bacteria were proposed for ground improvement, mainly via Enzymatically Induced Calcite Precipitation (EICP) to overcome these potential limitations (Ahenkorah et al., 2022). Whilst most of the current literature has been focusing on urease enzyme, using carbonic anhydrase (CA) enzyme appears to be an attractive alternative for ground improvement applications, as it could contribute to carbon sequestration while producing biocement (e.g., Russo et al., 2022). CA enzyme is with an active site that contains a Zinc ion (Zn^{2+}), whose primary purpose is to convert CO_2 and water into carbonic acid, protons, and bicarbonate ions. Six hundred thousand molecules of CO_2 can be hydrolysed per CA per second (Trachtenberg et al., 1999). However, the use of free enzymes in the soils, including CA, entails many challenges linked predominantly to the stability of the enzyme once released into the soil and also the limited enzyme supply (as there is no generation of more enzyme from the soil bacteria, unlike when microbial biocementation is used) and the poor reusability of the CA enzyme (Moon et al., 2020) which increases costs and reduces the sustainability of the technique. Previous studies have addressed these challenges by immobilising the

CA enzyme to improve the stability of CO_2 capture in bioreactors (e.g., Rasouli et al., 2022). Enzyme-metal nanoflowers have been extensively used in CO_2 capture technology because they exhibited higher enzyme activity and stability (Wang et al., 2013, Ge et al., 2013). The current work focuses on synthesising, characterising, and utilising hybrid nanoflowers for innovative delivery and enzyme stabilisation due to enhanced enzyme activity efficiency and studies factors affecting bioprecipitate production through material characterisation. This is the first study to formulate a hybrid CA enzyme and metal phosphate for biocementation and ground improvement application. The overall aim of the work is to improve ground properties while reducing CO_2 emissions in the construction industry.

2 MATERIAL AND METHODS

2.1 Synthesis of Metal−CA Hybrid

The metal−CA hybrid nanoflower assay was synthesised according to the protocol followed by Duan et al. (2018). For the synthesis, 0.6 mL of metal phosphate solution (120 mM) was added into 100 mL of phosphate-buffered saline (PBS, 0.1 M, pH 7.4) solution containing 10 mg of CA and incubated at 4°C for 2.5 days.

2.2 Carbonic anhydrase Activity Measurement

As in Martin et al. (2009), the activity of the CA enzyme was determined colourimetrically. Briefly, the activity for p-nitrophenyl acetate hydrolysis was determined at room temperature in a reaction mixture (1.35 ml) containing freshly prepared 3 mM p-nitrophenyl acetate in phosphate buffer (0.13M and pH 7.2). The reaction was allowed to proceed for 5 minutes, and the change to A_{348} per min was measured. Then the CA activity was characterised by the amount of p-nitrophenol produced per unit of time, and enzyme activity was expressed in terms of U.

$$\text{CA activity}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{(\Delta A_{348}\text{T} - \Delta A_{348}\text{B}) \times 1000}{5 \times Volume}$$

2.3 Bioprecipitation testing

Precipitation experiments were conducted using a total volume of 30 mL: Sodium Bicarbonate ($NaHCO_3$), Calcium Acetate ($Ca(CH_3COO)_2$), and water as control, free CA, and Cu-CA hybrid nanoflowers. The composition of each system is shown in Table 1. The total volume of the deposition system was 30 mL. Each precipitation experiment was mineralised in a conical flask at 25°C, 120 rpm, and the investigation lasted for 24 h. Precipitation experiments were conducted in triplicate.

Table 1. *Experimental conditions for precipitation*

	CA (mL)	CA-Cu (mL)	$(Ca(CH_3COO)_2$ (mL)	$NaHCO_3$(mL)
Control	0	0	10	10
Free CA enzyme	10	0	10	10
CA-Cu hybrid	0	10	10	10

2.4 Biocementation experiments

Biocementation of the soil was conducted in plastic syringes of diameter 25 mm and height 50 mm. The soil was dried, sterilised at 105°C, and hand packed into the syringe. For biocementation, water, CA, or CA-Cu hybrid nanoflower was injected and allowed to stand in the column for 2 h. After that, each column was fed with one pore volume of cementation solution of $CaCl_2$ and $NaHCO_3$ that were sequentially injected into the column. This process was repeated every 24 h for a period of 14 days. At the end of the experiment, the syringes with samples were cut open with a hot knife, and the sand columns were taken out to inspect the biocementation and tested for unconfined compressive strength.

2.5 Characterisation analysis

The pH and conductivity of each system were monitored using a Hanna Instruments HI 9813-6N Waterproof pH/EC/TDS Temperature Meter. The total amount of precipitate formed in each system was

measured by discarding the supernatant after centrifuging, and the biomineral dried and weighed. The total weight (M_1) of the empty bottle was measured. Then after the precipitation experiment, the bottle was centrifuged, and precipitate in the centrifuge bottle after drying was recorded (M_2). The total weight of biomineral formed (M_2-M_1) was the difference between the weight of the empty centrifuge tube and the final weight after drying in an oven at 105°C for 24 h. The microstructure of the biomineral formed was analysed using scanning electron microscopy (SEM) with Thermo Scientific Pharos FEG-SEM (ThermoFisher Scientific, USA) with high vacuum mode, 15KV acceleration voltage, elemental analysis done with Energy dispersive X-ray detector (EDS), and Raman spectroscopy (Horiba Aramis confocal Raman microscope,) with 633nm laser (1% power), 50X objective, 100um pinhole, 600l/mm grating.

3 RESULTS AND DISCUSSIONS

3.1 Synthesis of hybrid enzyme-metal nanoflower

The Cu-hybrid nanoflower flower-like structures were confirmed with SEM images shown in Figure 1. The resulting image from scanning microscopy showed good dispersity of the petals with three-dimensional microstructures like dahlia flowers. SEM-EDS mapping revealed that the structures consisted of Cu, N, O, and P. The Cu was derived from copper ions from the salts used. In contrast, the elemental N, O, and P originated from CA, as previously reported (Shende et al., 2018). The hybrid "nanoflowers" presented unified structural morphology like a "*Dahlia pinnata*", as depicted in the final image of Figure 1.

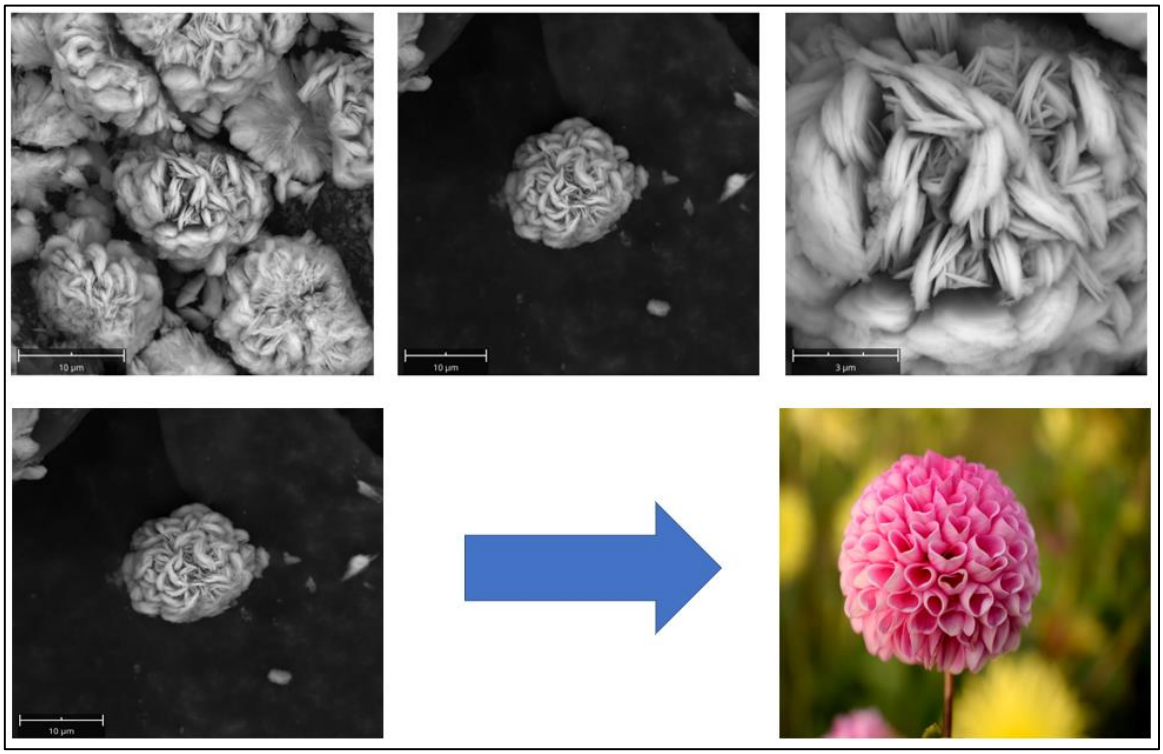


Figure 1: *SEM images of Cu-CA hybrid nanoflower at different magnifications (Photograph courtesy of public resources in the network)*

Previous researchers have documented the formation mechanisms (Altinkaynak et al., 2016; Duan et al., 2018) in a three-step chemical reaction of nucleation, growth, and completion. Figure 2 illustrates this mechanism where the Cu^{2+} ion reacts with phosphate groups to form copper phosphate as a primary crystal. Secondly, the primary crystal binds to amine groups on the protein backbone. Finally, the self-assembled protein-Cu^{2+} complex produces separate nano petals that act as a seed and grow into a nanoflower. This process is eco-friendly and easy to control, occurring at room temperature and pressure.

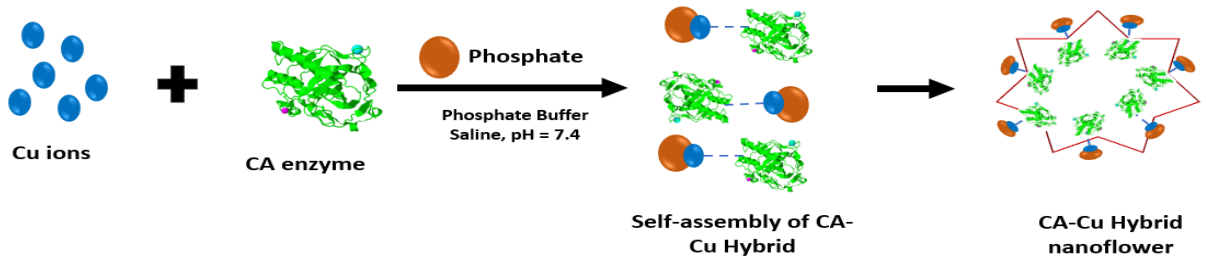


Figure 2: *Schematic illustration of the copper-carbonic anhydrase nanoflower formation process*

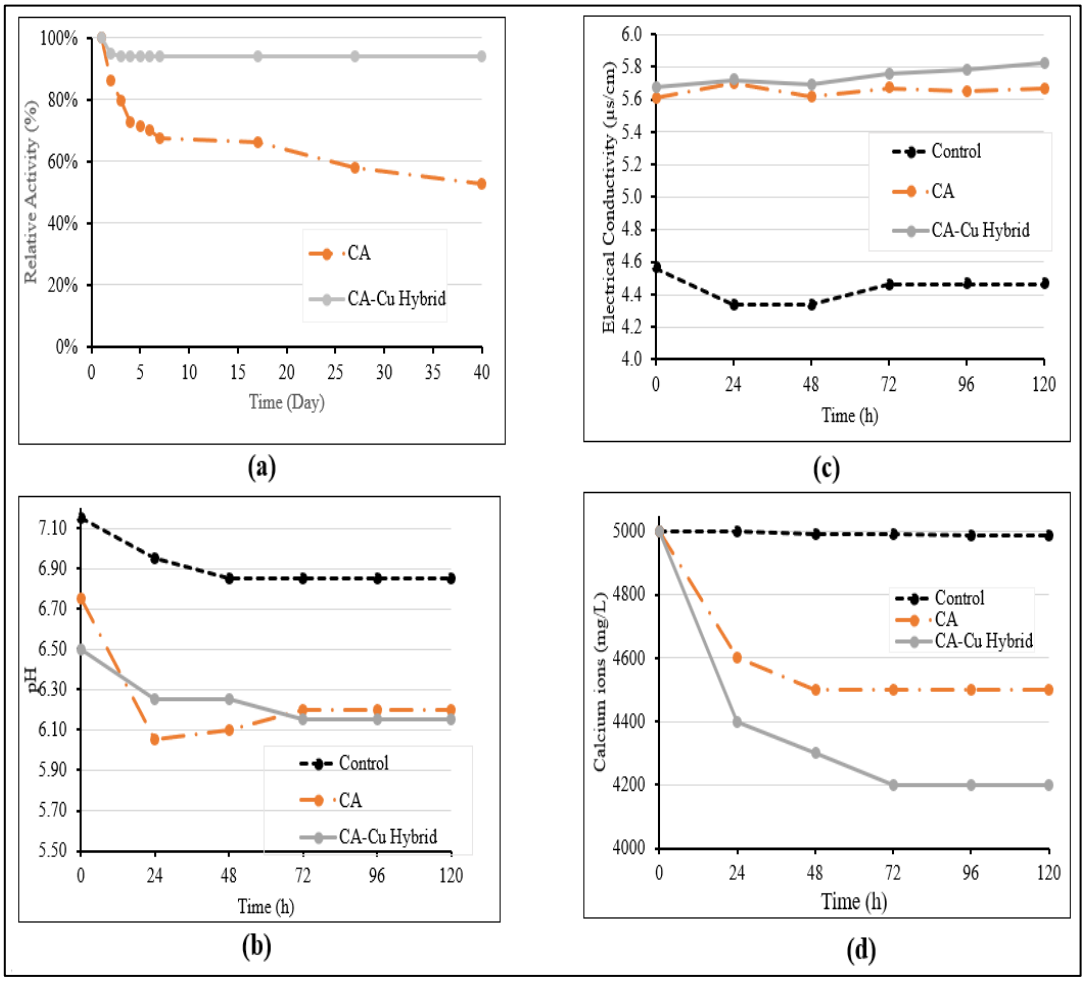


Figure 3 *(a) Stability of CA and CA-Cu hybrid over time (b)Electrical conductivity changed over time (c) the changed curve for pH (d) Change curve of Calcium concentration.*

3.2 Stability of CA-Cu and its CO_2 capture ability

The relative activity for both the free and immobilised enzyme was compared. The relative activity is the ratio of the retained activity of an immobilised CA or a free enzyme to its initial activity. The results showed that the immobilised efficiency was 15%. Similar studies on immobilisation have been found in similar studies for immobilisation CA. Despite the low immobilisation, the free CA enzyme lost 66% of its initial activity within 7 days when incubated in PBS (pH 7.4) at room temperature. Still, under the same conditions, CA-Cu hybrid nanoflowers maintained most of their initial activity of 94% even after one month (Figure 3a). This result shows that the immobilisation strategy lowers enzyme deactivation and can be used longer than previously reported (Zhang et al., 2021).

To understand the CO_2 capture of the synthesised CA-Cu and free CA, the water chemistry was investigated, and the pH (Figure 3b), conductivity (Figure 3c), and calcium ion content (Figure 3d) were

determined. As shown in Figure 3b, CA-Cu and free CA systems showed increased electrical conductivity compared to the water-only system. This difference in conductivity is due to the CO_2-H_2O system, where when CO_2 dissolution occurs in the water, there is a formation of HCO_3^- leading to increased conductivity. In the control samples with water only, the electrical conductivity and pH remained the same, as in the water-only system, the dissolution of CO_2 in water was limited. The results showed that immobilised CA is more stable and contributed to CO_2 capture and precipitation occurred, as previously reported (Giri & Pant, 2019). The reduction in calcium ion content is due to bioprecipitation as the CA captures CO_2 from the atmosphere and forms biominerals as opposed to water only, which did not show any changes in calcium concentration (Figure 3d).

3.3 Bioprecipitation

The bioprecipitation results showed the distinct difference in morphology between precipitates from purely chemical reactions and bioprecipitation, as shown in Figures 4a to 4c. Additionally, Raman spectroscopy identified the precipitates as calcite (Figure 4d). The peak at 288 cm^{-1} arises from the vibrations of the CO_3^{2-} groups that involve rotatory oscillations of those groups. The peaks corresponded to the lattice vibration of calcite and the in-plane bending vibration peak of the carbonate group of calcites at approximately 716 cm^{-1} and therefore identified as calcite. The results thus indicate that the CA enzyme can be used to biocement soil, as previously reported (Pan et al., 2019), due to calcite formation.

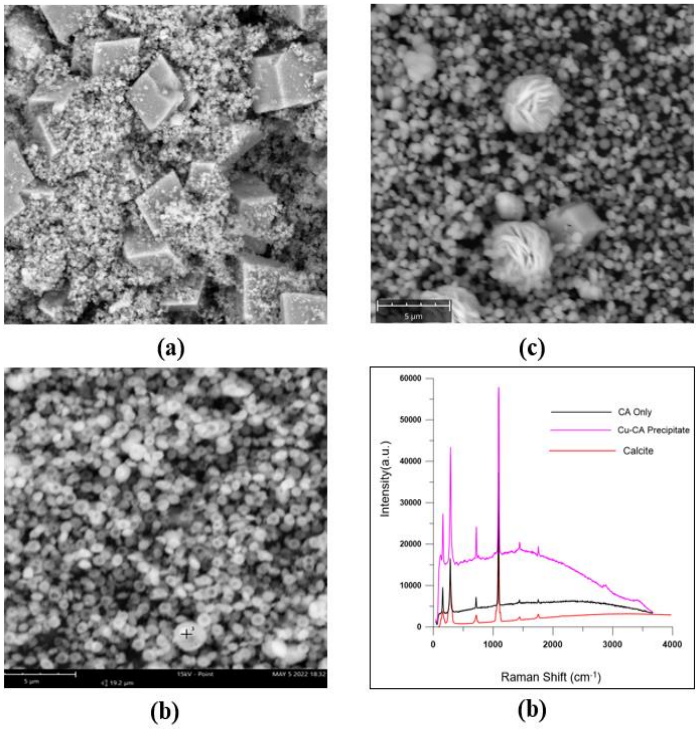


Figure 4 *(a)SEM image of chemical precipitation (b) SEM image of bioprecipitation with CA (c) SEM image of bioprecipitation with CA-Cu hybrid (d) Raman spectroscopy of CA and CA-Cu*

3.4 Biocementation of soil using formed nanoflower

Figure 5 shows the images of fine-grained soil specimens after biocementation treatment with cementation solution only, free CA, and CA-Cu. A pocket penetrometer was used to evaluate the improvement in the strength of the biocemented soil treated. A more detailed investigation of strength improvement is ongoing, but pocket penetrometer results indicated an unconfined compressive strength q_u of approximately 500kPa for free CA and approximately 1000kPa for hybrid CA-Cu, consistent with soil cementation. Conversely, the control sample (treated with cementation solution but no enzymes) crumbled, and q_u measurements could not be taken. The results show that the CA enzyme can biocement soil, as previously reported (Pan et al., 2019).



Figure 5 Typical image of a biocemented sand sample made from *(a) Cementation Solution only as control, (b)Cementation solution and CA enzyme, (c) Cementation solution and CA-Cu nanoflower.*

4 CONCLUSIONS

In this study, we investigated stabilised carbonic anhydrase (CA) enzyme delivery as one possible way to overcome the challenges of enzyme instability in the biocementation process. The study synthesised, characterised, and utilised inorganic hybrid nanoflowers, demonstrating their capability as a useful delivery alternative. Namely, the results showed that the bovine carbonic anhydrase enzyme enhanced the CO_2 hydration reaction, resulting in a bioprecipitation reaction and calcium carbonate production. Unconfined compressive strength values of a fine-grained soil measured by pocket penetrometer were approximately 500kPa for free CA and approximately 1000kPa for the hybrid CA-Cu. The scanning electron microscope micrographs and Raman Spectroscopy confirmed calcite as the primary precipitate formed, which can act as a bonding agent between soil particles for ground improvement applications. This shows promise that upon further development, this alternative method incorporating a carbon capture process can potentially be used for soil biocementation to increase the efficiency and overall sustainability of the process for geotechnical applications. The study and demonstration of the effect of precipitates on soil properties is the focus of the next stage of planned research on the topic.

5 ACKNOWLEDGEMENTS

The European Commission funded this work under the Horizon 2020 Marie Skłodowska-Curie Individual Fellowships Grant Number 101025184 (Grant holder: London South Bank University).

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