# Sustainable Development and Characterization of Biodegradable Collagen-Calcium Carbonate (Col-Caco3) Microporous Composite Scaffold For Bone Tissue Regeneration

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## Abstract

Microporous CaCO3 scaffolds were fabricated using the polyurethane (PU) sponge template method, incorporating various compressive ratios (95%, 75%, and 50%) and the presence or absence of additives to evaluate their effect on mechanical properties. The resulting CaCO3 scaffolds were then coated with collagen (COL) at room temperature. The microporous structure and mechanical properties of the produced biomaterials were analyzed using Field Emission Scanning Electron Microscopy (FE-SEM) and the Shimadzu Compact Tabletop Testing Machine (EZ Test), respectively. The results indicated that the inclusion of additives and the COL coating led to a reduction in porosity and an enhancement in the mechanical properties of the biomaterials. Notably, the most significant decrease in porosity was observed at a 50% compressive rate when additives were present. The composite scaffolds composed of CaCO3-COL with additives at this compressive rate exhibited a maximum compressive modulus of 10.78 MPa. Additionally, the highest fracture stress (253 KPa) and strain energy density (539 J/m³) were recorded in the composite scaffolds of CaCO3-COL with additives at a 75% compressive rate. These findings demonstrate that combining pure CaCO3 with collagen and additives significantly improves the mechanical properties of porous composite scaffolds, enhancing their suitability for sustainable bone tissue engineering applications.

**Keywords:** Scaffolds; Mechanical properties; Tissue engineering; Compressive ratio; Collagen; Sustainable

***Significance*** | Microporous COL-$CaCO_3$ scaffolds with additives and compression tuning significantly improved mechanical strength, supporting sustainable bone tissue engineering applications.

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## 1. Introduction

Currently, tissue engineering methods have enormous promise in biomedical applications. Tissue engineering technique is employed for bone regeneration, and bone grafting by growing mesenchymal stem cells (MSCs) on a porous scaffold for a set time and then transplanted into the injured region (Lu et al., 2011). Scaffolds work as a substrate to facilitate cell attachment and preservation of differentiated function without inhibiting proliferation. It is a template to organize and control the proliferation of cells and aid in creating extracellular matrix (ECM) (Thomson et al., 1995). This scaffold may be constructed utilizing either natural or synthetic polymers. Microscopically, bone is a very complex and specialized kind of connective tissue that gets calcified. A mineralized tissue, made of an organic matrix fortified by deposits of calcium phosphate crystals; in other words, bone is a natural composite

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material. Usually, collagen type-I fibers (about 95%), proteoglycans, and various non-collagenous proteins (5%) form bone organic matrix (Barrère et al., 2005). Bone mineral apatite is structurally and physically, unstable and extremely reactive that includes non-apatite carbonate and phosphate groups. This high reactivity provides certain physicochemical, biological, functional, and chemical features important in the formation and dissolution of the crystals in biological tissues (Barrère et al., 2005). Collagen (COL) is a structural protein that is said to be one of the most suitable materials for constructing artificial substitutes for diseased or damaged tissues and organs (Chvapil et al., 1973). COL-based scaffolds have been found to have superior biological performance owing to their high porosity and permeability (O'Brien et al., 2004). However, COL scaffolds generally suffer from weak mechanical characteristics and quick enzymatic breakdowns, hence restricting their usage when great mechanical strength is required (Moreau et al., 2009; Ng et al., 2004). Because of its good biological qualities, COL might be blended with other materials such as HA (hydroxyapatite) to increase its mechanical capabilities. Naturally porous calcium phosphate biomaterials have been recognized as appropriate scaffold options in bone tissue engineering and calcium carbonate biomineral might be employed for the production of biocompatible hybrid materials and templates (Moyo et al., 2004; Yang et al., 2002). Porosity is an important quality to consider for a porous biomaterial since it promotes the flow of nutrients, metabolic waste, and other materials during cell growth. All cells inside the scaffold must have a comparable access requirement, and this is generally done by creating a framework with a high degree of porosity of a suitable dimension (Sabree et al., 2015). Therefore, the purpose of this work was to build a microporous biomimetic COL-$CaCO_3$ scaffold by integrating $CaCO_3$ materials into PVA (polyvinyl alcohol). Further, the porosity of the produced pure $CaCO_3$ scaffold specimen was regulated by varying the compressive pressure (95, 75, and 50%) after removing the surplus slurry from the scaffolds to develop sustainable biodegradable collagen-calcium carbonate (COL-$CaCO_3$) microporous composite scaffold for bone tissue regeneration.

## 2. Materials and method

### *2.1 Synthesis of $CaCO_3$ porous Scaffolds*

$CaCO_3$ porous scaffolds were manufactured by employing a polyurethane (PU) sponge template approach. Calcium carbonate slurry was made using commercial Calcium carbonate powder (Wako Pure Chemical Industries, Ltd.). A combination of $K_2CO_3$ and $Li_2CO_3$ (Wako Pure Chemical Industries, Ltd.) with a molar ratio of 38:62 was added to $CaCO_3$ powder as an additive. Then this additive combination was applied to $CaCO_3$ powder in 0.7wt%. Calcium carbonate powder with or without addition was combined with polyvinyl alcohol (PVA) (Wako Pure Chemical Industries, Ltd.) 5wt% solution following the ratio of 1.66: 1 (1.66g $CaCO_3$:1ml PVA) using a centrifuge mixing machine (Imoto Co, Ltd.). PU sponge templates were cut into cubes and submerged into the prepared $CaCO_3$ slurry. Then the surplus slurry was removed by 95%, 75%, and 50% compression to prevent pore clogging and dried at room temperature for 24 hours to enable the $CaCO_3$ to settle nicely on the sponge framework. The calcination was done at 200° C for 6 h at a heating rate of 10° C/min and lastly, sintering at 500 °C for 3 hours to firm $CaCO_3$ porous scaffolds. These samples were classified as 'pure $CaCO_3$ scaffolds'. Fig. 1(a) demonstrates the schematic depiction of the sintering process of manufactured composite scaffolds.

### *2.2 Fabrication of $CaCO_3$ porous Scaffolds with Collagen*

The produced pure $CaCO_3$ porous scaffolds were created using Collagen-1 solution with or without addition. For manufacturing, prepared pure $CaCO_3$ porous scaffolds were dipped into the Collagen-1 solution and vacuumed at least for 30 min to remove the air. Excess collagen solution was removed by pipette manually and the samples were dried at room temperature for at least 48 hours. These samples were called '$CaCO_3$-COL coated scaffolds'. Fig. 1(b) displays the schematic depiction of the construction of pure $CaCO_3$ scaffolds with COL.

### *2.3 Characterization and analysis*

#### *2.3.1 Compression testing*

Compression tests were carried out by employing Shimadzu Compact Tabletop Testing Machine EZTest (EZ-S Series) configured with a 500N load cell and a crosshead speed of 1mm/min. Mechanical characteristics were examined in terms of compressive modulus, fracture stress, and strain energy density. Specimens acquired following the manufacturing process were submitted to research mechanical characteristics. Specimen dimensions of L (mm) in length, W (mm) in width, and H (mm) in height were measured before the test due to shrinkage following sintering. Firstly, force F (N) and displacement ΔH (mm) were obtained and then stress σ (MPa) (Equation 1) and strain ϵ (Equation 2) were computed. Elastic modulus E was derived from the first slope of the stress-strain curve before the breakdown of the specimen. For each condition, 6 samples were examined and the average result was reported.

$$Stress\ \sigma = \frac{F}{L \times W} \quad (1)$$

$$Strain\ \in = \frac{\Delta H}{H} \quad (2)$$

The porosity of the specimen was determined based on the density of prepared porous material and volume according to Landi *et al.* (2000) and Munar *et al.* (2006).

$$Porosity\ [\%] = \left(1 - \frac{Weight\ of\ HA}{(Volume\ of\ HA \times Density\ of\ HA)}\right) \times 100 \quad (3)$$

**Table 1.** Variation in porosities at different compressive rates of pure and fabricated composite scaffolds.

| Compressive rate (%) | Average porosity (%) | | | |
|---|---|---|---|---|
| | Pure $CaCO_3$ scaffolds (±3) | | Fabricated $CaCO_3$-COL scaffolds (±2) | |
| | With additive | Without additive | With additive | Without additive |
| 95 | 88 | 92 | 89 | 91 |
| 75 | 88 | 91 | 88 | 90 |
| 50 | 87 | 89 | 86 | 88 |

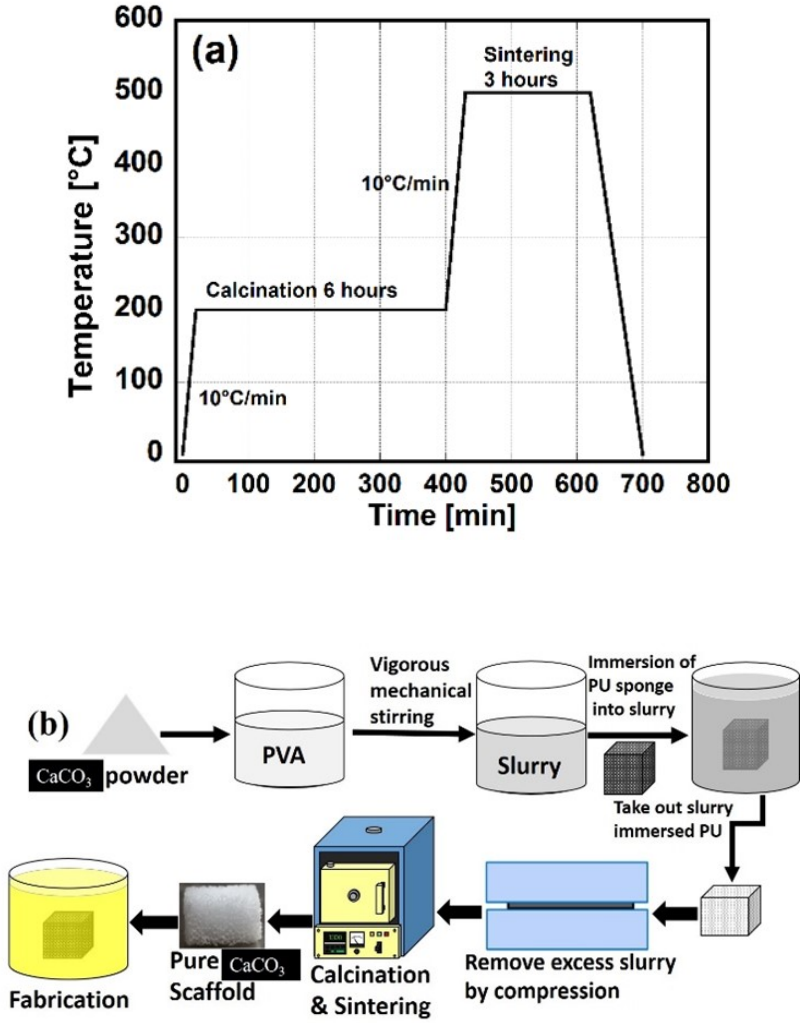


**Figure 1.** Schematic of (a) the sintering processes and (b) fabrication of CaCO3-COL composite scaffolds.

*2.3.2 Microstructural characterization*

FE-SEM (Field Emission Scanning Electron Microscope) is one of the most flexible material morphology characterization tools known. So, porous microstructures of produced biomaterials were evaluated by FE-SEM (Hitachi, Ltd. S-4100) according to Islam et al. (2012). To make specimens favorable for being studied by FE-SEM, specimens were put on the specimen holder affixed by using carbon tape and applying electro-conductive Dotite D-550 (Fujikura Kasei Co, Ltd.) at the bottom of the sample. Subsequently, specimens were coated with a thin coating of Pt/Pd-alloy using a vacuum sputter (Hitachi E1030 Ion Sputter) and subsequently, the analysis was done.

*2.4 Statistical analysis*

All data were reported as means ± standard deviation (SD) were generated from 6 separate samples. The analysis of variance (ANOVA) was used to evaluate the recorded data by using the Kaleida Graph (Synergy Software, Reading, PA, USA) and statistical analysis was performed by Fischer's Least Significant Difference (LSD) comparison test where any difference was considered statistically significant when the p-value was $< 0.05$.

**3. Results and discussion**

Typical FE-SEM micrographs of pure $CaCO_3$ scaffolds and manufactured composite scaffolds were carefully studied. **Figure 2** shows the FE-SEM micrographs of produced $CaCO_3$-COL composite scaffolds at 95%, 75%, and 50% compressive rate and sintered at 500 °C for 3 hours. Here, (a), (b); (c), (d); and (e), (f) revealed the FE-SEM micrographs of produced $CaCO_3$-COL composite scaffolds with or without additive for 95%, 75%, and 50% compressive rate, respectively. It was discovered that all of the manufactured composite scaffolds showed distinct microporous shapes. Microstructure pictures of FE-SEM analysis demonstrated that porosities were partly blocked or decreased by the integration of COL under coating conditions at all compressive rates. It was also discovered that the incorporation of additives blocked the porosities of the manufactured scaffolds homogenously while the porosities were uneven in the fabricated scaffolds without additives. Sous et al. and Shors found that the biomechanical characteristics of calcium phosphate ceramics rely on the degree of porosity (Shors et al., 1993; Sous et al., 1998) et al.,). It was interesting that our produced $CaCO_3$-COL composite scaffolds displayed extremely microporous characteristics with or without additives.

It was discovered that COL was spread across the pores of the pure scaffolds eventually creating a layer that boosted strengthening or load-bearing capacity or decreased the brittleness of the produced biomaterials. In previous work, it was observed that the construction of pure HA scaffolds with COL or COL/HA particles under 2-phase settings created a unique layer or phase, leading the manufactured scaffolds to be strengthened and their mechanical characteristics to be enhanced. Moreover, it was discovered that higher sintering temperatures significantly increased the mechanical characteristics of produced HA porous composite scaffolds by lowering porosity (Islam and Todo, 2016). It was shown from this study that the porosities of the fabricated composite scaffolds were dramatically decreased when constructed with COL by adding additives at a 50% compressive rate (**Table 1**). Previously, it was shown that scaffolds containing macropores in the outer layers allow access for cells, and blood vessels and stimulate new bone formation (Sudo et al., 1983). Werner et al. discovered that cells were only detected at the surface for 10% and 20% porosity showing a lack of interconnectedness between the pores (Werner et al., 2009). Therefore, manufactured microporous composite scaffolds with better mechanical stability under this investigation might be applied in bone tissue implant engineering.

Compression tests were conducted on samples from all scaffold designs manufactured at different compressive ratios. There was substantial diversity seen in the mechanical response of individual scaffolds chosen from each batch of scaffolds constructed. **Figure 3** demonstrates the impact of additive and compressive ratios on the mechanical characteristics of produced $CaCO_3$-COL composite scaffolds. It was seen that the inclusion of additive induced an elevation of modulus in virtually every specimen, but the maximum modulus (10.78 MPa) was recorded in $CaCO_3$-COL composite scaffolds with additive at 50% compressive rate sintered at 500 °C (**Fig. 3**).

Further, it was noted that when the compressive rate was reduced the obtained modulus was raised for every sample. It was believed that at 50% compressive ratio porosity was successfully decreased at maximum level to cause the enhancement of observed mechanical properties of the produced biomaterials (**Table 1**).

The fracture stress behavior of pure $CaCO_3$ and produced $CaCO_3$-COL composite scaffolds with or without additives were also studied. Effects of compressive ratio and addition of additive on the fracture stress of pure $CaCO_3$ and manufactured $CaCO_3$-COL composite scaffolds are depicted in **Figure. 4.** It was reported that highest fracture stress (253 KPa) was for $CaCO_3$-COL composite scaffolds with additive at 75% compressive rate followed by 242 and 208 KPa for $CaCO_3$-COL composite scaffolds with additive and without additive, respectively at 50% compressive rate. On the other hand, the lowest fracture stress (66 KPa) was detected for pure $CaCO_3$ scaffolds without additives at a 95% compressive rate. So, it was ensured that fracture stress greatly improved in produced composite scaffolds.

Strain energy density was also studied for pure $CaCO_3$ and produced $CaCO_3$-COL composite scaffolds with or without addition as a mechanical parameter. Variation of strain energy density of pure and constructed scaffolds with or without additive are displayed in Fig. 5. It was discovered that maximal strain energy

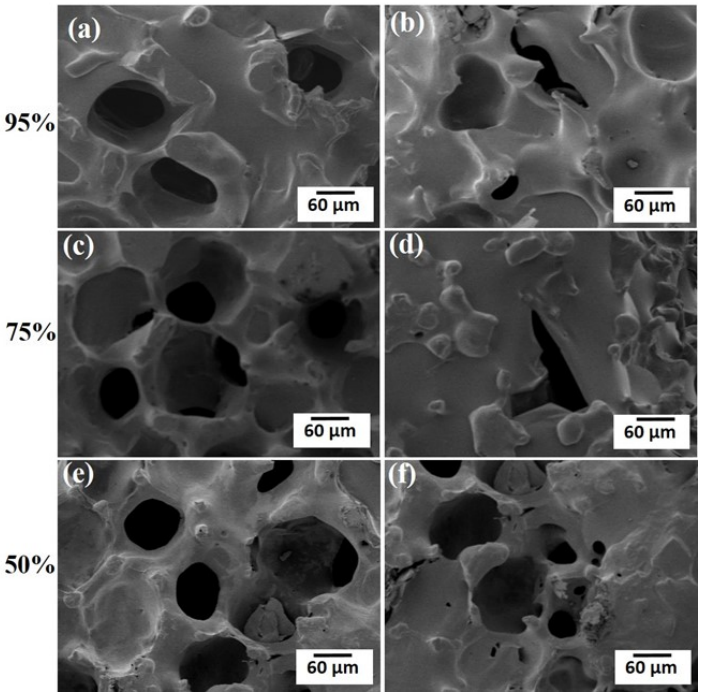


**Figure 2.** FE-SEM porous microstructures of fabricated CaCO3-COL composite scaffolds at various compressive rates. Here, microstructures of composite scaffolds with or without additives are denoted by (a), (b); (c), (d); and (e), (f) for 95, 75, and 50% compressive rate, respectively.

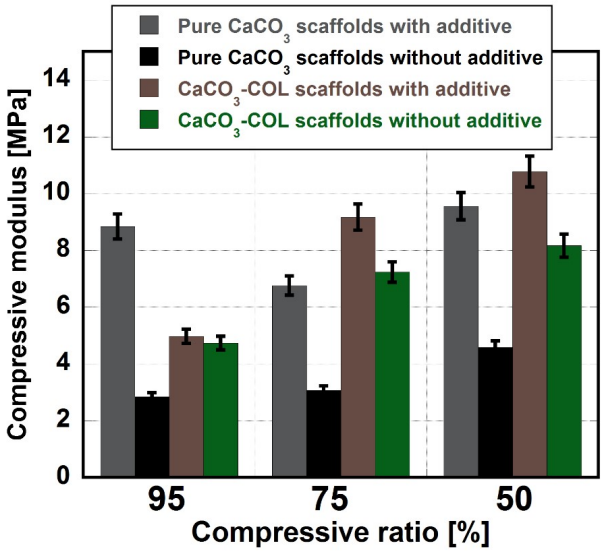


**Figure 3**. Effects of addition of additive and compressive ratio on the compressive modulus of fabricated composite scaffolds.

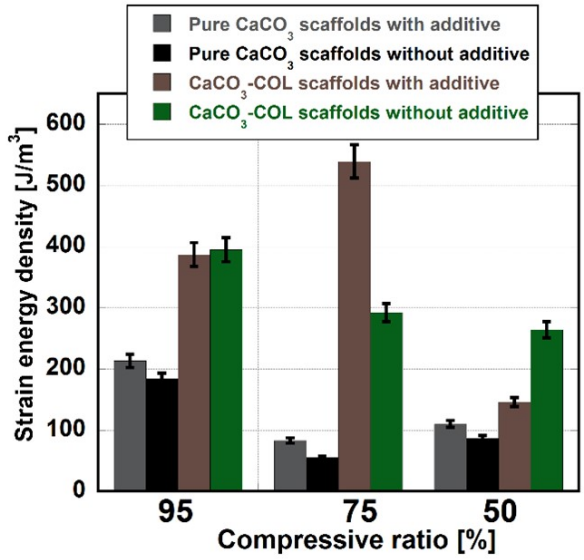


**Figure 5.** Effects of addition of additive and compressive ratio on strain energy density of fabricated composite scaffolds.

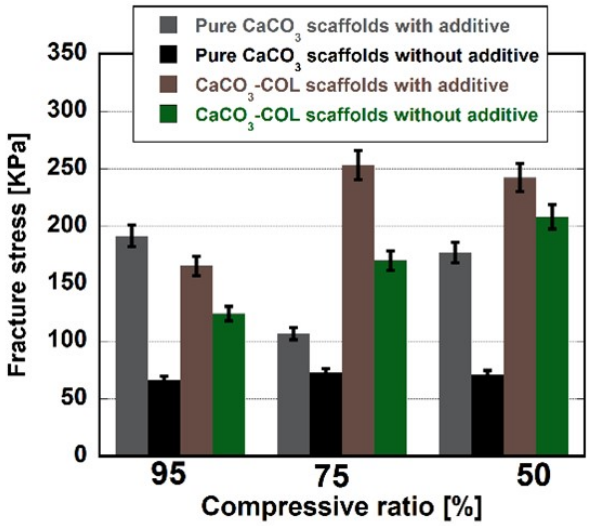


**Figure 4.** Influences of addition of additive and compressive ratio on the fracture stress of fabricated composite scaffolds.

density of produced $CaCO_3$-COL composite scaffolds with additive was 539 J/m3 at 75% compressive rate sintering at 500 °C. Further, 395 J/m3 and 387 J/m3 strain energy density was detected for produced $CaCO_3$-COL composite scaffolds with and without additive, respectively at 95% compressive rate. The lowest strain energy density (55 J/m3) was found at 75% compressive rate for pure $CaCO_3$ scaffolds without addition. Therefore, it was obvious that creation of pure $CaCO_3$ scaffolds by COL with addition of additive greatly enhanced the mechanical characteristics of the produced biomaterial.

A successful bone regeneration requires a scaffold to have acceptable mechanical characteristics and porous structure, until the implanted cells or the regenerated tissues get accustomed to the surrounding environment. When the scaffold has considerably poorer mechanical characteristics than the surrounding tissue, it might severely distort or shatter, and appropriate tissue regeneration will not occur. So, it is vital to construct a scaffold with adjustable mechanical characteristics and with adequate porosity architectures to provide the mechanical stability with sufficient tissue development throughout the regeneration process (Park and Todo 2011).

Any synthetic biomaterials should cover any materials or systems suggested for therapeutic applications to replace part of a live system or to operate in close contact with living tissues. In this context, typical biomaterials are developed and manufactured readily and have already found uses in tissue engineering (Cen et

al., 2008). Under this investigation for manufacturing $CaCO_3$ composite, Type-1 collagen was integrated which is a good scaffold material for developing artificial replacements for tissue engineering (Wang et al., 2003). In our previous study, it was found that porosity was effectively reduced to the maximum level at a 50% compressive ratio and further, an in vitro stem cell study showed significant cell adhesion and proliferation over the fabricated HAp-COL and HAp-COL/HAp composite scaffolds (Islam et al., 2019). Therefore, these findings demonstrated that production of pure HAp using COL and COL/ HAp materials exhibited improved impacts on the mechanical characteristics of manufactured porous composite scaffolds. However, to the best of our knowledge, this is the first report of the creation of biomaterial employing solely $CaCO_3$ for bone tissue engineering. Hopefully, biological assessment of manufactured composite scaffolds will happen very shortly.

## 5. Conclusion

The current study focused on the development and fabrication of microporous $CaCO_3$ scaffolds with COL at different compressive ratios with or without additives. The effects of various compressive pressures and additives on the mechanical properties of the fabricated composite scaffolds were evaluated. The results indicated that the fabrication of pure $CaCO_3$ scaffolds with COL and adding additives at 75% and 50% compressive ratios demonstrated the best performances. Further, the biological properties of the fabricated composite scaffolds will be evaluated near future in our laboratory.

## Author contributions

M.S.I. and M.J. selected the title and study objectives, P.K. and R. A. conducted the literature review and references management, M.S.I. & J.R. wrote the manuscript, and A.K. and G.M.S.R. revised the manuscript. All authors reviewed and approved the final manuscript.

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## Competing financial interests

The authors have no conflict of interest.

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