Novel polydopamine/halloysite nanotubes reinforced brushite calcium phosphate cement for bone regeneration with synergistic regulation mechanical/osteogenic capacity

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1. Materials and methods

1.1. Materials

Halloysite (pharmaceutical grade) and 2-(3,4-dihydroxyphenyl)ethylamine hydrochloride were purchased from Energy Chemicals. (Tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$, 600-900 mesh, 96%, medical grade, 3A) and monocalcium phosphate monohydrate (MCPM, $Ca(H_2PO_4)_2•H_2O$, 98%, 3A) were purchased from Energy Chemicals.)

1.2. Preparation of HNTs@PDA

Take 5 g of **HNTs**, add it to a round-bottom flask, then add 200 mL of distilled water, put in a magnetic stir bar, and ultrasonically stir for 30 minutes. Take out the tris solution and 2-(3,4-dihydroxyphenyl)ethylamine hydrochloride from the refrigerator and let them reach room temperature. Then add 2 g of dopamine. Adjust the pH value to alkaline and stir for 48 h. Centrifuge, wash with water, and obtain a precipitate at 8000 r/min. Freeze-dry to obtain dry **HNTs@PDA**.

1.3. Transmission electron microscope (TEM)

HNTs and **HNTs@PDA** were dispersed in an ethanol solution and sonicated to achieve a homogeneous suspension, respectively. Aliquots of the sonicated suspension were then deposited onto a copper grid, followed by drying. The morphology and high-resolution imaging of the samples were conducted using a JEOL JEM-F200 transmission electron microscope (TEM) operated at an acceleration voltage of 200 kV.

1.4. Thermal gravimetric analysis of HNTs@PDA

Thermal gravimetric analysis (TGA) measurement was carried out under nitrogen on Perkin-Elmer Pyris 6 TGA (heating rate of 10 ℃/min) to record TGA curves.

1.5. Transmission electron microscope (TEM)

To synthesize Ca-HNTs and Ca-HNTs@PDA materials. 0.01 g of HNTs and 0.01 g of HNTs@PDA were added to $CaCl_2$ solution (3 mol/L, 2 mL), respectively. The mixtures were stirred for 24 hours to allow for the interaction between the HNTs/ HNTs@PDA and $CaCl_2$. Subsequently, the products were subjected to thorough washing and centrifugation with distilled water to remove excess calcium ions. After drying, Ca-HNTs and Ca-HNTs@PDA were obtained.

Ca-HNTs and Ca-HNTs@PDA were dispersed in an ethanol solution and sonicated to achieve a homogeneous suspension, respectively. Aliquots of the sonicated suspension were then deposited onto a copper grid, followed by drying. The morphology and high-resolution imaging of the samples were conducted using a JEOL JEM-F200 transmission electron microscope (TEM) operated at an acceleration voltage of 200 kV. Additionally, energy dispersive X-ray spectroscopy (EDS) with a JED-2300T energy spectrum model was employed to perform elemental analysis of the samples.

1.6. Preparation of CPCs

CPCs are composed of two calcium phosphate powders as the solid phase and the liquid phase. The solid phase is composed of β-TCP and MCPM (ratio 6/4) and **HNTs** or **HNTs@PDA**. The liquid phase is a 0.5 M citric acid solution. The liquid-solid ratio is 0.7 mL/g. The samples are represented as **Bru-CPCs**, **Bru-CPCs/1.5%HNTs**, and **Bru-CPCs/1.5%HNTs@PDA** respectively. Stir the bone cement sample in a glass beaker and then fill it into a cylindrical polytetrafluoroethylene mold (4.6 mm × 6 mm). After 5 minutes of solidification, take out the cement sample and incubate it at 37 °C and 100% relative humidity for 2 days.

1.7. Compressive strength of CPCs

Compressive strength could be defined as ultimate compressive stress when the specimens fracture. In this study, the compressive strength of CPCs was tested in a Universal Tester (Shandong Wanchen Testing Mechine Co., LTD, CMT2503) and the crosshead speed was 0.5 mm/min according to ISO13779-1.[1-3] CPC samples were uniformly polished on both sides before the test. Five CPCs samples (a diameter of 4.6 mm and a height of 6.0 mm) were tested for each group and during the testing the test environment temperature was maintained at 23 ± 2 °C. The compressive strength was finally expressed as mean ± SD.

1.8. Setting time

Setting time of the cement pastes was determined by a Gilmore apparatus pursuant to ASTM C266-13. The Gilmore apparatus included an initial setting indenter (113.4±0.5 g, Φ2.12±0.05 mm) and a final setting indenter (453.6±0.5 g, Φ1.06±0.05 mm).1 After the solid powder was mixed with the setting liquid, the paste was transferred into a mold (a diameter of 7.5 mm and a height of 15.0 mm) and placed at 37 oC and 98% relative humidity. The indenters were lowered vertically on the cement surface for 5 s to determine the setting time at intervals of 1 min until the paste was hardened. Initial and final setting occurred when there was no a complete cyclic penetration using corresponding indenters. Each test was repeated six times.

1.9. Weight loss and pH measurement

Each CPCs was put in ·the oven at 100 ℃ for 20 min and the weight was recorded as W_0. The sample was placed into SBF (the ratio was 0.1 g/mL) at 37 ℃ for different incubation time (0.5, 1, 3, 7 and 14 days). At the end of each prescribed time, the CPCs samples were collected and dried at 100 ℃ for 20 min. The dry weight was recorded as W_x (x = l,·2, 3, 4,·5, 6). The weight loss was calculated by the equation

Weight loss (%) = $(W_0-W_x)/W_0$

The pH value of the SBF solution at each prescribed schedule time was measured by a pH meter (PHS-2F, INESA Scientific Inc, China). All data was collected from 3 independent samples.

1.10. Scanning electron microscopy (SEM)

The fractured surfaces of CPCs were collected by SEM (Phenom Prox, Phenom China). All the samples were pre-coated by Au-Pd coating and then observed by SEM.

1.11. Porosity of CPCs

The porosity of CPCs was determined via a method of liquid displacement with water (ρ = 1.0 g/cm^3). Briefly, a scaffold with a mass of W1 and regular shape of V was immersed in a measuring cylinder containing distilled water. Pores of the scaffold were then completely filled with water via the evacuation process. Outer surface water was removed, and the mass of the water-impregnated scaffold was recorded as W2. The porosity of the scaffold was calculated as follows equation[4]:

porosity (%) = (W2-W1)/ρV

1.12. Anti-washout ability of CPCs

The anti-washout property of CPCs was measured by a shaking method. The solid powder was mixed with a setting liquid to obtain single, homogenous pastes. The pastes were transferred into a 2.5 mL syringe immediately after mixing and manually injected into phosphate buffered saline (PBS) solution. The petrie dishes were placed in a shaker (60 rpm) at 37 °C until the paste was hardened. The anti-washout property of the paste was determined if the paste did not visibly disintegrate during shaking. Optical appearance of the cements was photographed by Huawei nova8 mobile phone camera.[1, 5]

1.13. Cell culture

The mouse embryonic osteogenic precursor cells (MC3T3-E1) were purchased from Procell Life Science&Technology Co., Ltd. (Wuhan, China). 10% fetal bovine serum (Biological Industries, Israel) and 1% penicillin-streptomycin solution (Solarbio, China) were added to α-MEM (Biological Industries, Israel) basal medium to configure complete medium (BM). MC3T3-E1 cells were cultured in BM, a normal cell culture environment (37°C, 5% CO_2, 95% air), and when the cell density reached more than 80%, the cells were digested with trypsin solution (EDTA) and passaged. In the osteogenic induction study, osteogenic induction medium (OIM) was prepared by adding 10 mM β-glycerophosphate (Sigma-Aldrich), 50 μM ascorbic acid (Sigma-Aldrich), and 100 nM (Sigma-Aldrich) dexamethasone to BM, and MC3T3-E1 cells were first cultured in BM, and then the medium was switched to OIM when the density of the cells reached 70%-80%, and all of the cement specimens used in this study were sterilized by ultraviolet light for 4 h.

1.14. Cell adhesion and cell viability

MC3T3-E1 was inoculated on 4.6 mm × 6 mm × 1 mm tablet samples per cement set in 24-well tissue culture plates with 3×10^4 cells per well. MC3T3-E1 was cultured in BM and normal cell culture environment (37°C, 5% CO_2, 95% air). After 24 h, the BM was removed, and the cells were washed 3 times with PBS and then fixed with 4% paraformaldehyde at room temperature for 15 min. The cells were dehydrated with graded ethanol (30%, 50%, 70%, 90%, 95%, and 100%) for 5 min twice at each concentration and then freeze-dried. The cells were coated with gold and tested using a scanning electron microscope (Pharos, Phenom World BV).

Cell viability was determined by cell proliferation assay and live/dead staining. MC3T3-E1 cells were seeded in 96-well plates at 5×10^3 per well, cultured overnight in incubators, **HNTs** and **HNTs@DPA** were sonicated in complete medium at concentrations of 1 %, 1.5 % and 2 %, respectively, and then the medium in the 96-well plate was replaced, and after 24 h of culture, the original medium was removed and washed with PBS. Then the medium containing CCK-8 (Solarbio, China) reagent was added, and the absorbance value was measured at 450 nm wavelength by Multiskan Spectrum Microplate Spectrophotometer (Thermo Scientific) after incubation in the incubator for 2 h in the dark. CCK-8 reagent (Solarbio, China) was used to detect cell proliferation. CPCs (0.2 g/mL) were immersed in α-MEM medium containing 10% FBS and incubated at 37°C for 24 h. Then cement extracts with an extraction ratio (sample surface area/volume) of 1.25 cm^2/mL were obtained according to ISO 10993. Each well (96-well plates) was 5×10^3 cells and incubated by adding 10% fetal bovine serum α-MEM medium. after 24 h, the cell culture medium was replaced with the cement extract. After another 1, 2, and 3 days of incubation, each well was replaced with fresh BM and 10 μL of CCK-8. After another 3 h of incubation in the cell culture incubator, the absorbance was measured at 450 nm using a Multiskan Spectrum microplate spectrophotometer (Thermo Scientific). Cell culture medium without extract was used as the control.

MC3T3-E1 was inoculated as described above with 5×10^4 cells per well in 96-well tissue culture plates. After 24 h of incubation, the cell medium was replaced with cement extract and incubated for another 24 h, the Live/DeadViability/Cytotoxicity Kit (beyantine, C2015M) was used according to the manufacturer's instructions. Briefly, after removing the cell culture medium, the cells were washed twice with PBS, and live and dead cells were stained by adding calcein AM/PI, respectively. After incubation at 37°C for 30 min, the solution was removed, washed three times with PBS, and tested by confocal microscopy (FV3000 LCM, OLYMPUS).

1.15. Osteogenic differentiation

MC3T3-E1 was co-cultured with sterilized cement samples in BM with 1×10^5 cells per well in 6-well plates. The BM was changed daily. after the cell density reached about 80%, the medium was changed to OIM to induce MC3T3-E1 cell differentiation. After 7 days of incubation in OIM, the OIM was discarded and the cells were washed twice with PBS. Differentiation of MC3T3-E1 was detected by ALP activity.

ALP activity was detected with an ALP activity assay kit (Solarbio, BC2145). After 7 days of incubation, cells were lysed and ALP assay was performed with reference to the kit protocol (Beyotime Biotechnology). Total cellular protein was also determined by BCA protein assay kit (thermo fisher scientific). ALP activity results were normalized by total cellular protein. Medium without cement samples was used as a control.

ALP expression of MC3T3-E1 on day 7 was stained with BCIP/NBT kit (Beyotime, China) according to the manufacturer's instructions. The OIM was removed, washed 3 times with PBS, MC3T3-E1 was fixed using 4% paraformaldehyde solution for 30 min, washed again 3 times with PBS, and stained with 250 μL of BCIP/NBT solution for 15 min, and the images of the stained samples were captured with an inverted microscope.

1.16. Expression of the osteogenesis-related genes

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect MC3T3-E1 osteogenesis-related genes after cement application of OIM.MC3T3-E1 was co-cultured with sterilized cement samples in 6-well plates with 1×10^5 cells per well in BM. After the cell density reached approximately 80%, the medium was changed to OIM to induce MC3T3-E1 differentiation. After 7 and 14 days of co-culture, cell samples were collected and total RNA was extracted from the cell samples using the Easyspin RNA Rapid Tissue Cell Kit (RA105-01, BMBiomed, China). Reverse transcription of RNA into

complementary DNA (cDNA) was performed using the PrimeScript RT Agent Kit with gDNA Eraser (Takara, Japan) was used for detection. The expression level of Runx2 gene was detected by qRT-PCR. TB Green series (Takara Japan) was used Real time. primer sequence information is shown in **Table S1**. All quantifications were normalized to the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative gene expression levels were determined by the $2^{-\Delta\Delta Ct}$ method. These steps were performed in a PCR machine (Thermo ABI7300 real-time PCR machine, New York, NY, USA).

Table S1. The sequences of genes (F: forward primer; R: reverse primer)

Gene-Primer	Primer sequences (5' to 3')-F	Primer sequences (5' to 3')-R
Gapdh	CGGGGTCCCAGCTTAGGTTC	ATCCGTTCACACCGACCTTC
Runx2	TCACCTTGACCATAACAGTCTTCA	GGCGGGACACCTACTCTCAT
SPP1	AGCAAGAAACTCTTCCAAGCAA	GTGAGATTCGTCAGATTCATCCG
Col-1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
IBSP	CAGGGAGGCAGTGACTCTTC	AGTGTGGAAAGTGTGGCGTT
BgIap	GGCAATAAGGTAGTGAACAG	CAAGCCATACTGGTCTGATA

1.17. Statistical analysis

Quantitative data were provided as the mean±standard deviation (SD). Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Tukey's post-test comparison (GraphPad Software 8.0, USA). In all cases, the results were considered statistically significant with p value < 0.05 (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

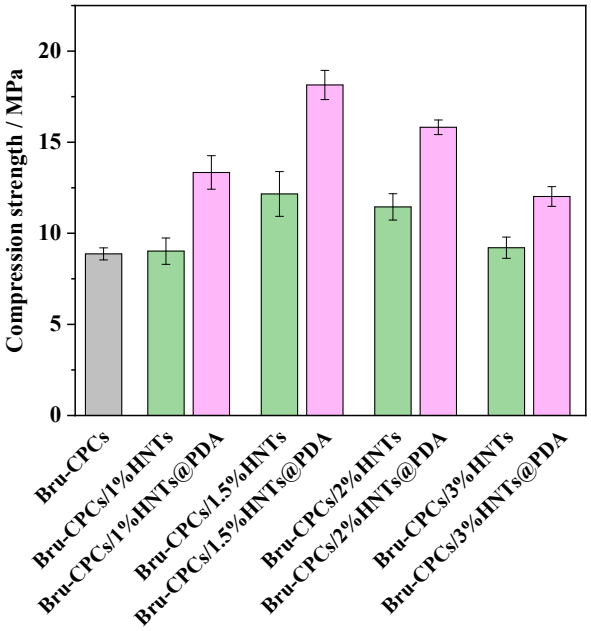

Figure S1. Compressive strength of CPCs after incubation in 37 °C and 100% relative humidity for 2 days

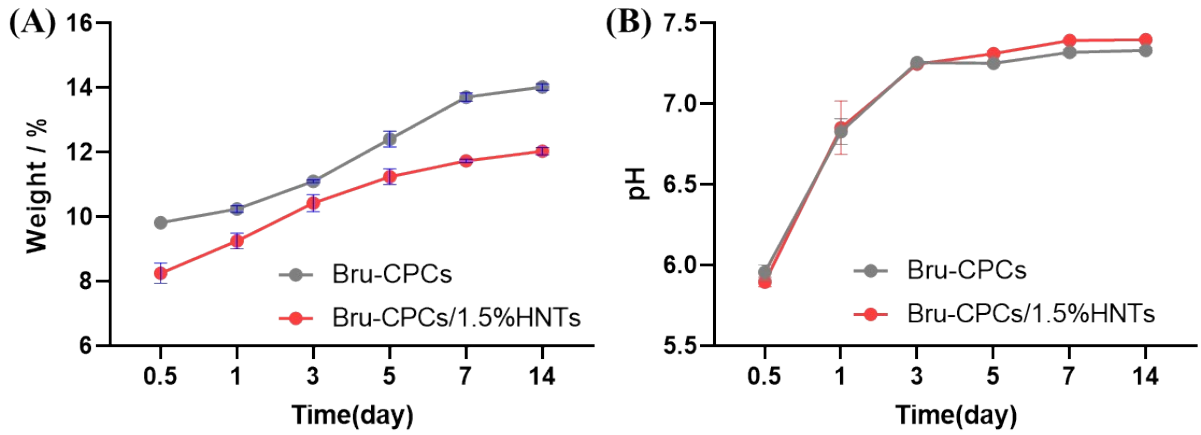

Figure S2. (A) Weight loss and (B) pH test of various CPCs.

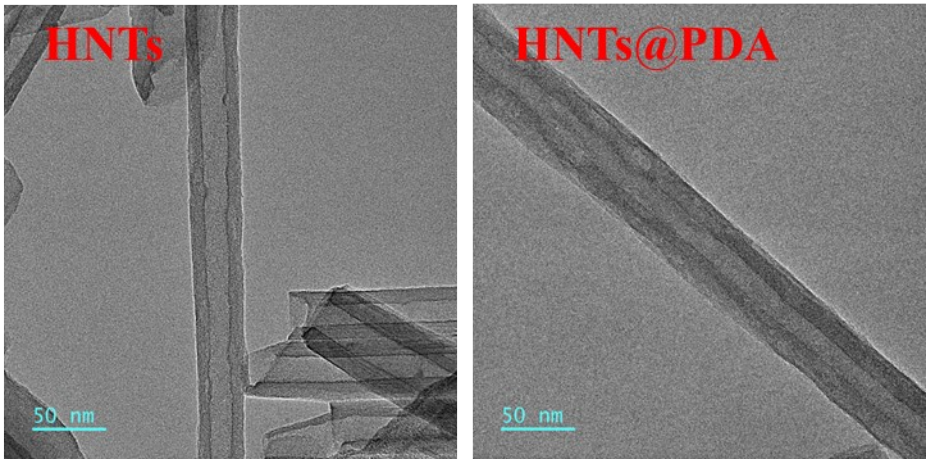

Figure S3. TEM images of **HNTs** and **HNTs@PDA**.

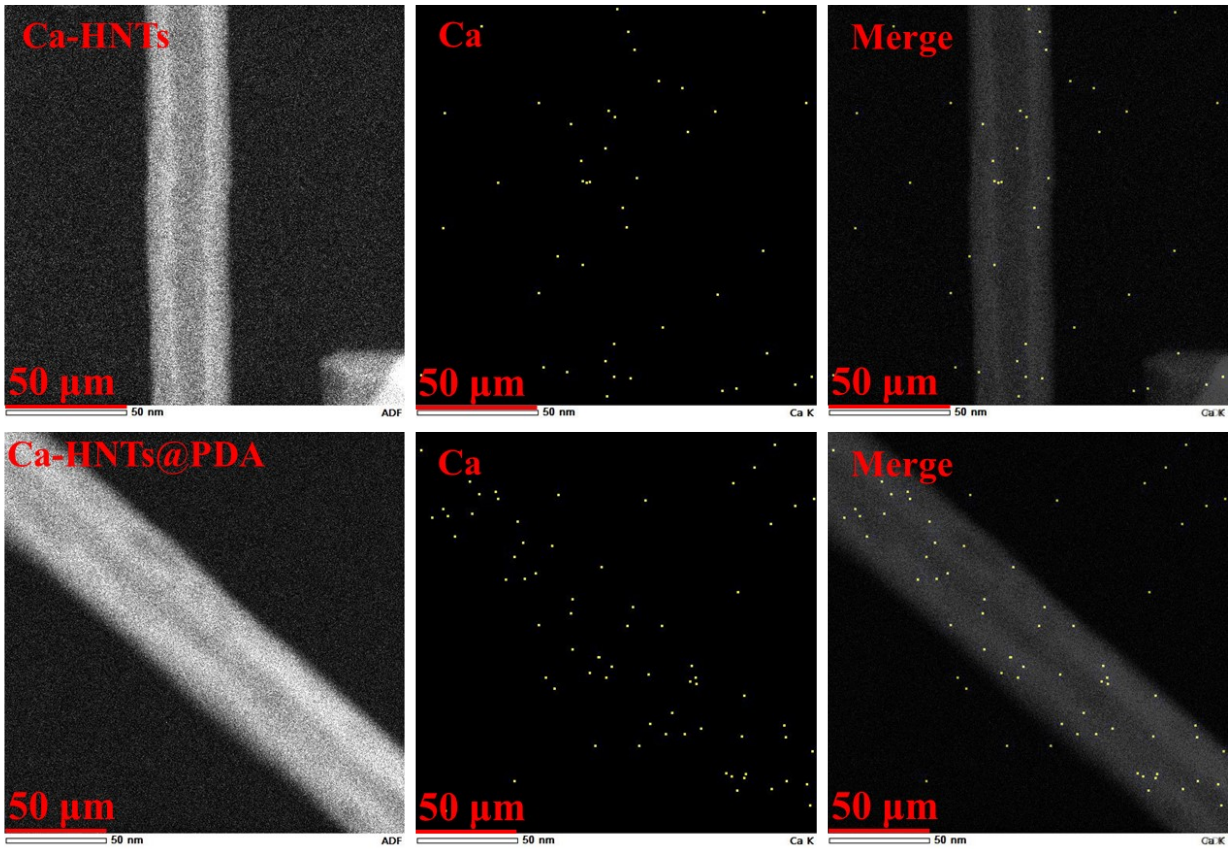

Figure S4. TEM-EDS elemental mapping image of calcium.

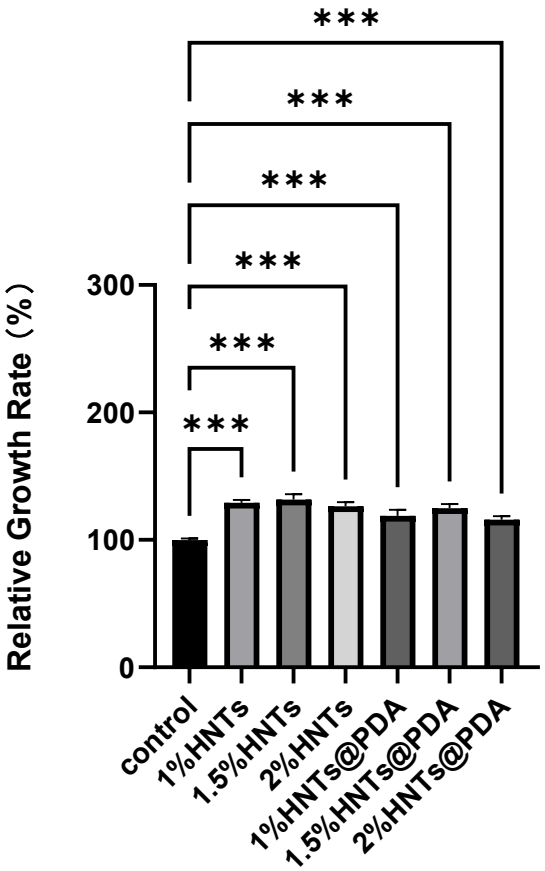

Figure S5. Cell viability of **HNTs** and **HNTs@PDA**, CCK-8 test (*P＜0.05, **P＜0.01, ***P＜0.001)

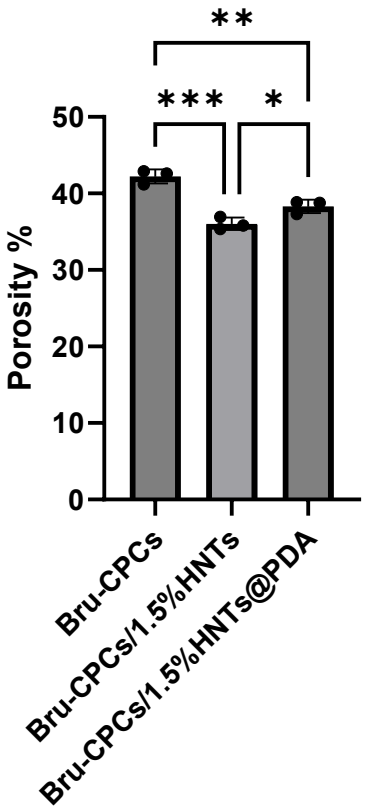

Figure S6. Porosities of CPCs.

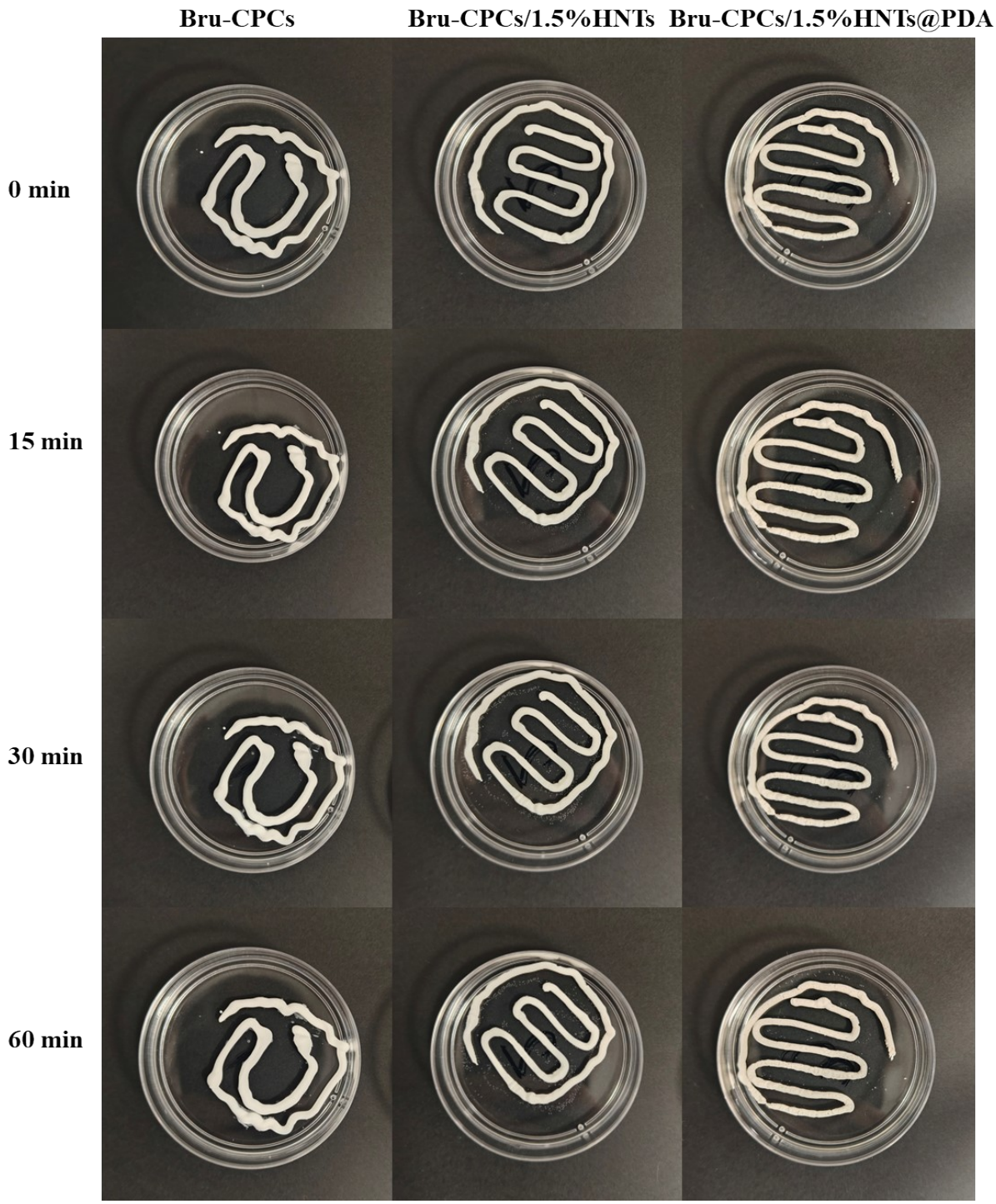

Figure S7. Anti-washout property of CPCs. Optical digital images of CPCs samples after injected into PBS solution and shaken at a speed of 60 rpm at different time.

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