Supplementary Information for:

Choline phosphate functionalized surface: protein-resistant but cell-adhesive zwitterionic surface potential for tissue engineering

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1. General

Materials: 2-Chloro-1, 3, 2-dioxaphospholane-2-oxide (COP, 95%) and 2-bromoisobutyryl bromide (BIBB) were purchased from TCI (China). 1, 1, 4, 7, 7-Pentamethyldiethylenetriamine (PMDETA, 98%) was purchased from J&k Chemical Ltd. (Beijing, China). 2-Dimethylamino-2-propyne was purchased from Alfa Aesar. Chromatographically pure tetrahydrofuran (THF) was purchased from Merck (Bombay). Chromatographically pure methanol and acetonitrile were purchased from Tedia Company (USA). *N, N*-dimethylformamide (DMF) was purchased from Tianjin Bodi Chemical Co., Ltd. (China). All other chemicals were of analytical grade or higher, purchased from Chengdu Kelong Chemical Reagent Plant (China). CuBr was purified by stirring for overnight in acetic acid, filtered, then washed promptly with absolute ethanol and vacuum-dried. Glass slides (18 × 18 mm, CITOGLAS®), cell counting kit-8 (CCK-8) assay kit, Dulvecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Baoxin Biotechnology Co. Ltd. (Chengdu, China). Ultrapure water with resistivity 18.2 MΩ·cm was used throughout.

Characterizations: ^{1}H NMR (400 MHz) spectrum was conducted using a Varian UNITY INOVA-400 spectrometer. Fourier transform infrared spectroscopy (FTIR) was conducted using a Nicolet Nexus 6700 FTIR spectrometer 156 (USA). X-ray photoelectron spectroscopy (XPS) data was obtained from 159 ESCALAB 220i (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The static water contact angle was measured with a contact angle goniometer (DSA100, Dataphysics, Germany) equipped with video capture. Confocal laser scanning microscopy (CLSM) was performed on a TCRS SP5 microscope (Leica, Germany).

2. Synthesis of prop-2-ynyle choline phosphate (p-CP)

All the glasswares were flame dried and protected by argon. Methanol (8 mmol) and triethylamine (8 mmol) were dissolved in a 5 mL THF (chromatographically pure). After cooling the solution down to − 20 ℃, COP (8 mmol) dissolved in THF (5 mL) was added slowly to the stirred solution over 1 h. The temperature of the reaction mixture was maintained at − 20 ℃ for 3 h and allowed to slowly warm up to room temperature for another 4 h. Triethylammonium chloride precipitate was filtered off and washed with THF.[1] The filtrate was evaporated under vacuum to give the residue as yellow oil. Then the residue was dissolved in 10 mL of acetonitrile (chromatographically pure) and cooled to − 20 ℃. 2-dimethylamino-2-propyne (16 mmol) was rapidly added to the solution. The reaction mixture was stirred for 16 h at 55 ℃, and subsequently, the mixture was precipitated into THF to obtain yellow viscous liquid (Yield: 36%).

^{1}H NMR data of p-CP (400 MHZ, methanol-d4, δ, ppm): 4.34 (CH≡C-C***H***$_2$, 2H), 3.95-3.98 (-OC***H***$_2$, 2H), 3.68-3.71 (-NC***H***$_2$, 2H), 3.59, 3.62 (-OC***H***$_3$, 3H), 3.26 (-C≡C***H***, 1H), 3.23 (-C***H***$_3$, 6H); FTIR spectrum (cm^{-1}) : 1250 ($\nu_{P=O}$), 2120 ($\nu_{C\equiv C}$).

3. Surface modification of glass slides

3.1 Clean the glass slides

Briefly, glass slides (18 × 18 mm) were first treated with "piranha" solution (a mixture of 70% concentrated sulfuric acid and 30% hydrogen peroxide) at the boiling temperature about 30 min. The slides were finally rinsed with a large amount of Milli-Q water and dried in vacuum oven for 2 h. [2]

3. 2 Immobilization of BIBB on the glass slides

The glass slides were immersed into 30 mL of CH_2Cl_2 (chromatographically pure) containing 1 mL of triethylamine (chromatographically pure), into which 1 mL of BIBB was dropwise added under gentle shaking in an ice-water bath. The reaction mixture was maintained at 0 ℃ for 2 h and left at room temperature for another 12 h. The bromo-terminated glass slides (Glass-Br) were cleaned with CH_2Cl_2, acetone, alcohol and Milli-Q water under ultrasonication for each 15 min, and then they were dried in vacuum oven.[3]

3. 3 Preparation of azido-terminated on glass slides

The Glass-Br slides were immersed in a saturated solution of NaN_3 in DMF, heated at 80 ℃ for 48 h. The slides were finally rinsed with a large amount of Milli-Q water and dried in vacuum oven to obtain Glass-N_3.[4]

3. 4 Surface "click reaction"

For surface treatments, solution of the p-CP were prepared (1 mM) in DMF. The Glass-N_3 slides were exposed to the DMF solution in the presence of PMDETA (0.1 mM). Finally, fresh CuBr (0.1 mM) was added under nitrogen flow, and then the flask was sealed and placed in an oil

bath at 60 ℃ for 3 h. The glass slides were rinsed with amounts of water followed by a rinse in 1% v/v NH_4OH and Milli-Q water to remove excess copper [5] and finally obtain Glass-CP.

4. Protein adsorption

The protein adsorption experiments were carried out with bovine serum albumin (BSA, 1 mg/mL in PBS, pH 7.4), collagen (1 mg/mL in PBS, pH 7.4) and 100 % fetal bovine serum (FBS). Glass slides were immersed in protein solution for 3 h at 37 ℃ or 100 % FBS for 3 h at 37 ℃ with an atmosphere of 5 % CO_2 and 95% relative humidity. After protein adsorption, the glass slides were carefully washed with PBS for three times. Then adding 1mL of 2 wt. % sodium dodecyl sulfate (SDS) solution for 30 min at 37 ℃ to remove the proteins adsorbed onto the glass slides. The amount of protein eluted into the SDS solution was quantified with commercial BCA Protein Assay Kits (Beyotime, Shanghai, China) at 578 nm.

5. Cell culture

5.1 Cell adhesion assay

In order to study the interaction between cell and CP-modified glass slides, CLSM image was used to observe cell adhesion. The slides were disinfected within 75% ethanol for 1 h and sterilized by UV irradiation for 1 h before use. The HUVECs were allowed to adhere for 3 h onto the glass slides in 6-well plates at a density of 5×10^4 cells/cm^2 in serum-free DMEM at 37 ℃ with an atmosphere of 5 % CO_2 and 95% relative humidity. After 3 h, the slides surface were washed three times with PBS solution to remove the loosely attached cells, then the slides were transferred in fresh 6-well plates and incubated in 2.5 mL DMEM supplemented with 10% FBS,

100 units/mL of penicillin and 100 μg/mL of streptomycin at 37 ℃ for 24-48 h with an atmosphere of 5 % CO_2 and 95% relative humidity. For CLSM image, at different time intervals, the culture media was removed and the cells were washed three times with PBS, the cells were fixed with 4% paraformaldehyde at 4 ℃ for 20 min, followed by being permeabilized with 0.1% Triton X-100 in PBS for 5 min and then rinsed with PBS three times. Subsequently, cells were incubated with 1μg/mL phalloidin-TRITC (sigma) in PBS at room temperature for 30 min to label the filamentous actins (F-actins), and rinsed with PBS three times. To label vinculin, the cells were incubated with a 1:100 dilution of monoclonal anti-vinculin-FITC antibody (Sigma-Aldrich) for 2h, and rinsed with PBS three times. After that, the cell nuclei were stained with DAPI (5 μg/mL) for 10 min. Finally, the glass slides were placed on a glass microscope slide and the prepared samples were subjected for fluorescence imaging by CLSM. The expression of integrins with cell adhesion in initial 3h was detected by immunohistochemical StreptAvidin-Biotin Complex (SABC) staining method.

5.2 Cell proliferation assay

Cell counting kit-8 (CCK-8) assay was done to analyze cell adhesion and proliferation under different experiment designs. Typically, the optical densities (OD) values of the CCK-8 containing culture media at 450 nm were measured after cultivating for 3 h at 37 ℃ in serum free or 10% FBS containing cell culture medium. After the CCK-8 was detection for cell culture 3 h, the medium was changed with DMEM supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin for CCK-8 assay of 24 and 48 hours. The cell culture experiments of initial 5 h attachment condition and the serum-preconditioned surfaces were also carried out, which were described in detail after Figure S4 and S5, respectively.

6. Statistical analysis

Data are presented as mean ± SD. Analysis of variance and *t*-test were used for statistical comparison. A *P* value < 0.05 is considered significant.

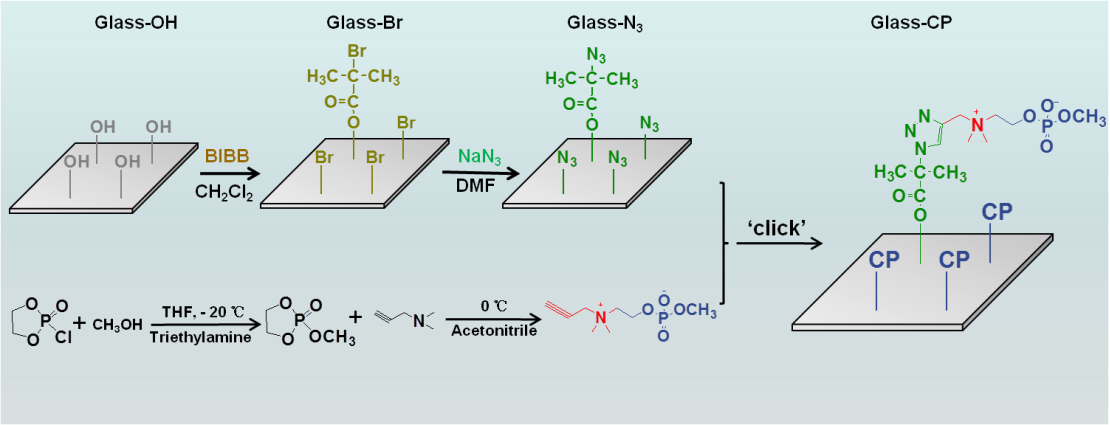

Figure S1. Synthetic route of CP-modified glass surface.

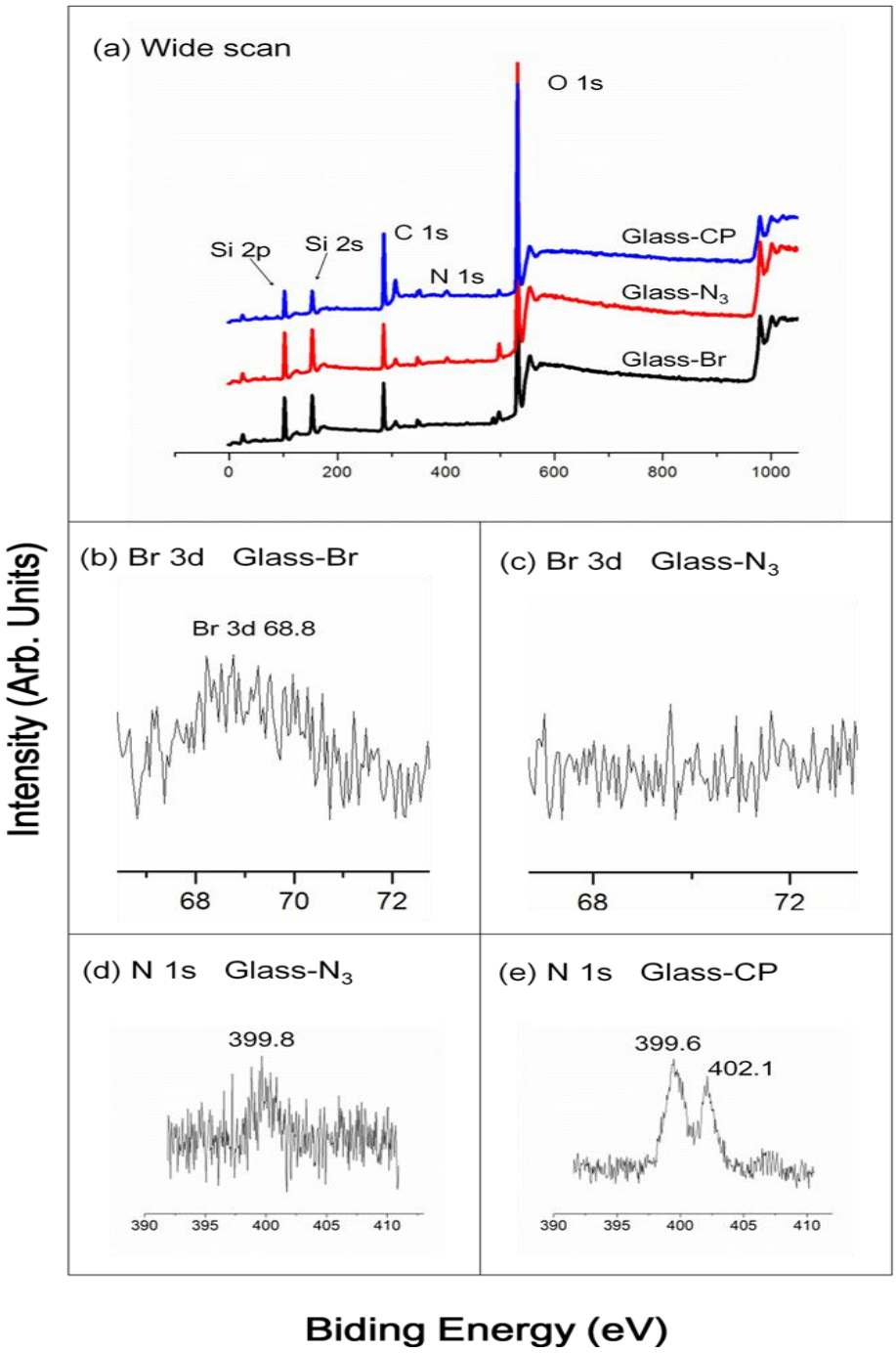

Figure S2. XPS results showing the wide scan (a), Br 3d (b and c), and N 1s (d and e) spectra of Glass-Br, Glass-N_3 and Glass-CP.

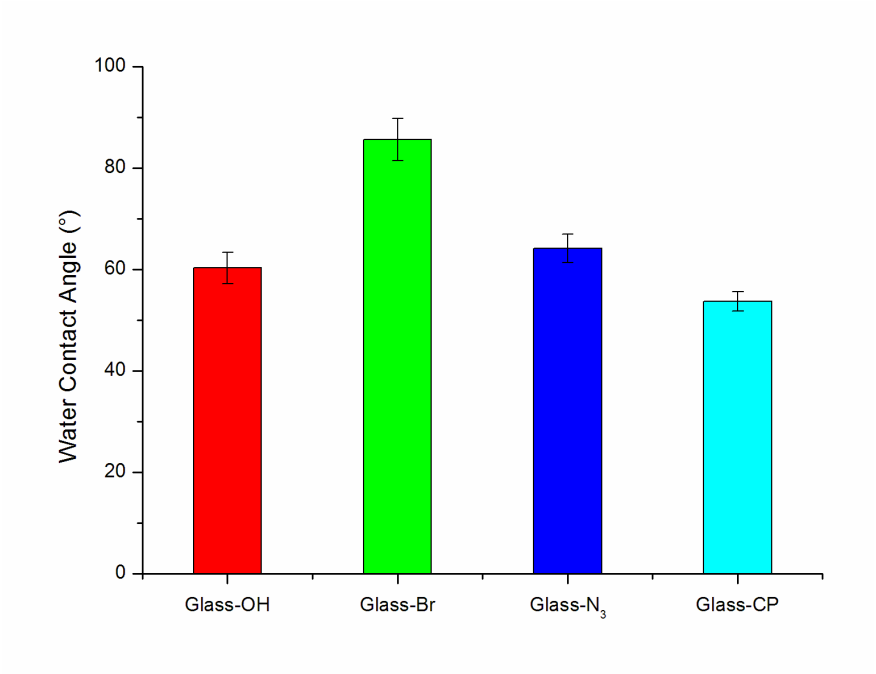

Figure S3. Data of water contact angle measurement.

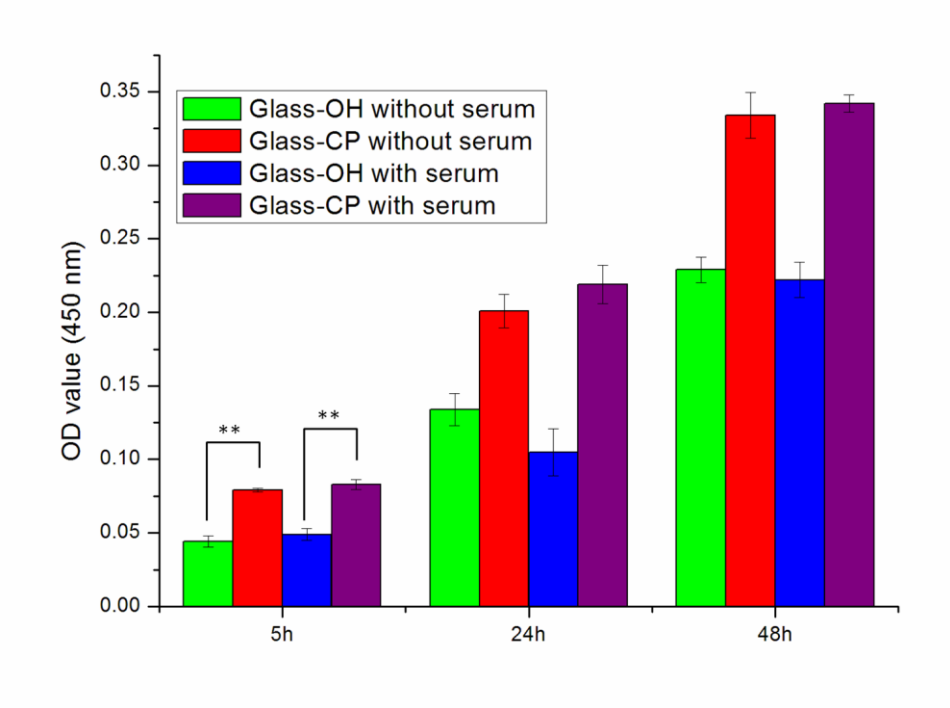

Figure S4. CCK-8 assay indicating the proliferation profiles of HUVECs, which were cultured without or with serum in initial 5 h. (** $p < 0.01$) ; Data is represented as mean ± SD, n > 3)

A complementary cell counting kit-8 (CCK-8) assay was used to test the adhesion and proliferation profiles of HUVECs on Glass-OH and Glass-CP (Figure S4). In the initial 5 h, the glass substrates were incubated in serum-free or serum-containing cell culture medium to explore the mechanism. As can be seen, cells significantly adhere more on Glass-CP than on Glass-OH, no matter in serum-free or serum-containing condition ($p < 0.01$). However, while compared with the results in the 3 h experiment (Figure 2), there is no significant difference between serum-free and serum-containing condition of cell adhesion on Glass-OH surface, thus the 5 h experiment is not a suitable time-point to reveal the mechanism although it also exhibits a better cell adhesion behavior of Glass-CP surface.

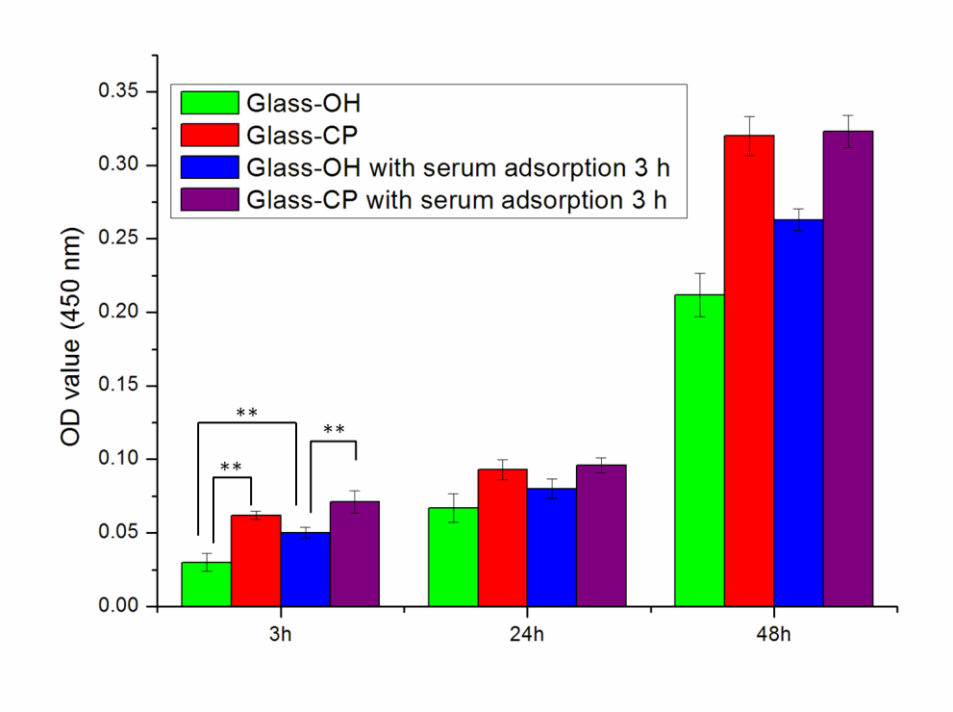

Figure S5. CCK-8 assay indicating the proliferation profiles of HUVECs, which were cultured after without or with serum protein adsorption in the initial 3 h. (** $p < 0.01$); Data is represented as mean ± SD, n > 3)

Another cell adhesion experiment was carried out after serum protein adsorption on surfaces for 3 h (Figure S5). In the initial 3 h, the optical density (OD) values of Glass-OH group after serum protein adsorption are significantly higher than that without serum protein adsorption ($p < 0.01$), indicating more serum protein adsorption and more cells adhered on Glass-OH surface, which exposed to serum containing media for 3 h before cell seeding. However, as for Glass-CP group, there is no significant difference of cells adhesion between with or without serum protein adsorption before cell seeding and the OD values are also higher than that of Glass-OH group.

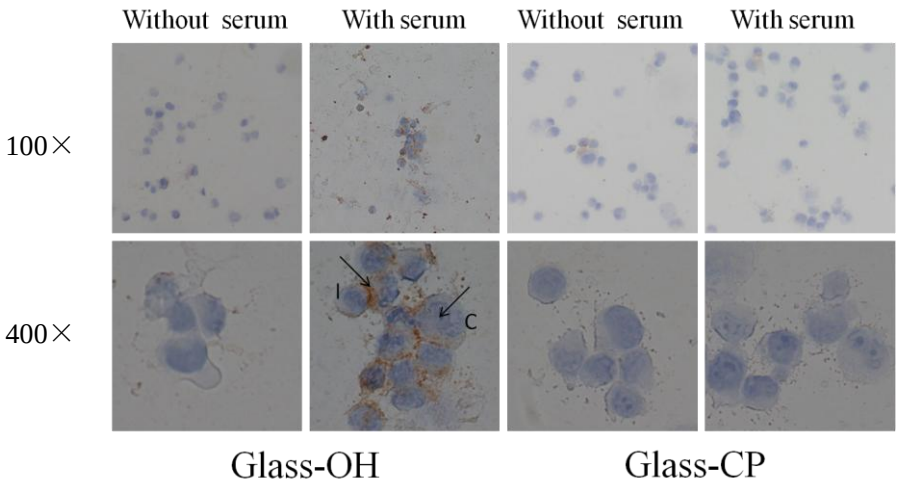

Figure S6. Immunostaining of integrins after HUVECs were cultured without or with serum in initial 3 h (I, integrins; C, cell nuclei).

The results of immunostaining of integrins β1 show that the expression of integrins of HUVECs adhesive on Glass-CP surfaces is weak positive no matter whether cultured with or without serum in initial 3h (Figure S6). However, when HUVECs adhesive on Glass-OH surfaces, the expression of integrins are positive and strong positive when cultured without or with serum, respectively. Therefore, it demonstrates that the cell-adhesive mechanism of Glass-CP is different since there is no expression of integrins β1, which mediate the adhesion of cell with extracellular matrix through ECM proteins.

References

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