Microporosity mediated proliferation of preosteoblast cells on 3D printed bone scaffolds

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Abstract
Microporous structure plays a significant role in bone tissue engineering, to influence inductive bone formation and elevate bone ingrowth inside microporous scaffolds. We hereby fabricated microporous scaffolds by 3D printing with mixture of porogens and photopolymerizing resin. Our method can tune the microporosity of scaffolds from 0% to 36% by varying the porogen concentration in the inks from zero to 60 vol%. The microporosity and micropore size of scaffolds can affect in vitro expansion of preosteoblast cells. We evaluated the attachment, spreading and proliferation of MC3T3-E1 mouse preosteoblast cells on the printed porous scaffolds. Our studies revealed that preosteoblast cells’ in vitro adhesion and proliferation were significantly mediated by the printed scaffolds. Cells proliferation on scaffolds with 20%–30% microporosity showed much higher rate than on other scaffolds. In this microporosity range, scaffolds also showed much better cell spreading and morphologies. At a low level of microporosity (<7%), the cell proliferation rate was even lower than the solid scaffolds, which indicates that the microporous structure is “toxic” for cells at low microporosity range. A higher microporosity (>35%) led to very poor cell attachment and is unfavorable for the proliferation of MC3T3-E1 cells. Furthermore, the printed dual macro-/micro-porous scaffolds showed higher proliferation rate of MC3T3-E1 cells as compared to mono-pore sized scaffolds, either the macro-porous or microporous structure.

Keywords
3D printing, additive manufacturing, bone tissue engineering, microporosity, vat photopolymerization

1 INTRODUCTION

Annually, millions of bone transplants are performed worldwide.11 Traditional autografts and allografts have been used for decades, but they have many limitations, including the limited size and supply, the risk of disease transmission and immune rejection.2,3 Alternative methods have been developed to fabricate synthetic bone grafts to promote regeneration of feasible healthy bone. Many synthetic alternatives with potential osteogenesis, made of polymers, metals, ceramics, and composites, have been tried as artificial scaffolds for bone tissue engineering but
with limited success. The ideal bone scaffold should be biocompatible and osteoconductive, with osteoinductive factors to enhance new bone ingrowth. Among these factors, porosity is one of the most critical one in improving the osteogenesis of scaffolds for bone tissue engineering, as both cell seeding and bone ingrowth are well developed with high porosity. The bone scaffold should have a similar structure to that of natural bone, consisting of cancellous bone and cortical bone. Cancellous bone, also called trabecular or spongy bone, is the internal tissue of the skeletal bone, and has an open cell porous network with a 50–90 vol% porosity. Cortical bone is the dense outer layer of bone, with a porous structure, but have a lower porosity than cancellous bone. Macroporosity (pore size > 100 μm) is usually required to facilitate the osteogenesis and angiogenesis, while microporosity (pore size < 10 μm) plays a significant role in enhancing the osteoinduction and elevating bone ingrowth inside the microporous scaffolds. Open and interconnected pores are necessary to allow nutrients and molecules to transport to the core of the bone grafts, and facilitate cell ingrowth, vascularization, as well as waste material removal.

Since the porosity of the bone scaffold is very important, many manufacturing techniques and biomaterials were used to fabricate the scaffold with controlled porosity. Molds or post-processing combining with gas foaming, gel casting, or emulsion are typically used. Although some progresses have been achieved to make porous scaffold for bone tissue engineering, difficulties still remain for control of scaffold architecture that including pore size, interconnectivity, and porosity. Recently, with the fast development of the advanced manufacturing (AM) technology, more and more research demonstrated that AM technology is one the promising method for bone tissue engineering, especially for treatment of complex and critical-sized bone defects. AM technology, sometime called as three dimensional (3D) printing, is a method to fabricate 3D objects in a layer-by-layer manner. 3D printing is well known for its flexibility in creating objects with complex structures and for applications that require personalization.

Various 3D printing techniques combing emulsion templates, particle/salt/sugar leaching, phase inversion, thermally induced phase separation, particle sintering, solvent or non-solvent methods, et al have been developed to create patient-specific scaffolds with porous architectures and mechanical properties that are suitable for cellular infiltration, vascularization, tissue formation. Porogen templating is one of the most powerful technique to use as a template for generation of the desired porous material biomedical materials and devices. Many efforts to combine injection 3D printing techniques with porogen templating have been attempted to build the desired 3D morphologies and porosity. Surprisingly, although digital light processing (DLP) printing is a faster and higher quality printing technique than the injection processing, there are few reports of using porogen templating in DLP printing process. The main problem may come from the instability of the mixture of resin and porogens, together with a difficulty to control the porosity. In the present work, we demonstrated the successful 3D printing of porous bioscaffolds by combing the DLP printing and porogen templating technique. Most importantly, we studied the influence of porogen concentration on the microporosity of 3D printed scaffolds. Our results showed that the in vitro attachment and proliferation of preosteoblast cells were remarkably mediated by the microporosity of the printed scaffolds, which provides an important guidance for future bone scaffold design and manufacturing.

2 | RESULTS AND DISCUSSION

2.1 | 3D printed microporous scaffolds

Di(ethylene glycol) diacrylate (DEGDA), a hydrophilic crosslinking monomer to form a PEG like polymer, was chosen as the photopolymerization resin for DLP printing because they have been proven to have good biocompatibility and widely used as carriers for drug delivery and various other biomedical applications. Hydrophilic DEGDA have a capability to form stable porogen templates with some polar solvents. The solubility of monomers in porogenic solvents have a great influence on the onset of phase separation of polymers formed from the starting solution during the free radical photopolymerization. Herein, a ternary mixture of 1-Octanol, ethanol and water was selected as porogens, in which 1-Octanol and ethanol can dissolve DEGDA, but not the polymer formed by DEGDA. Water was used as co-solvent to help to enlarge micropores in the scaffolds. Firstly, the resin and the porogen mixture was mixed with the volume fraction (fVP) of 0–60 vol%, and homogenized before printing (Figure 1). The micropores result from the phase separation between porogens and resins during the early stages of the polymerization.

Four different structures were printed on a DLP printer for different purposes as shown in Figure 2, Figure S1 and S2. The cubes with dimensions of 10×10×10 mm (Fig S1) were used to measure the density and microporosity of the 3D printed scaffolds. The bowl shape scaffolds with a flat bottom and honeycomb-structured scaffolds were designed for cell culture (Figure 2A). The printed diamond structures declare that the inks with porogens can be used to print complex structure by DLP 3D printing (Figure 2B).
Herein, different types of scaffolds (named as PG0, PG10, PG20, PG30, PG40, PG50, and PG60), from the inks with different $f_{VP}$ (0 vol%, 10 vol%, 20 vol%, 30 vol%, 40 vol%, 50 vol%, and 60 vol%, respectively), were obtained, in which bowl-shaped scaffolds were named as bPG0, bPG10, bPG20, bPG30, bPG40, bPG50, and bPG60, respectively; honeycomb-structured scaffolds were named as hPG0, hPG10, hPG20, hPG30, hPG40, hPG50, and hPG60, respectively. Scaffold printed with neat DEGDA showed a contact angle of 61°, suggesting a hydrophilic wettability. Furthermore, an increasing of the wettability was observed with increasing the $f_{VP}$ (Table S1). The microporosity was controlled by varying $f_{VP}$ of porogens in the resin. With increasing the $f_{VP}$, the color of the printed objects changes from transparent to translucent and finally to white opaque due to light scattering from porous structures (Figure 2, Figure S1 and S2). SEM images in Figure 3 displayed the surface morphologies and microporous structures of scaffolds. The pore size in the scaffolds increased with the $f_{VP}$ in the inks. When the $f_{VP}$ is 0–20 vol%, no pores (Figure 3A) or the nanoscale pores (Figure 3B) are shown in the SEM images. While the $f_{VP}$ is up to 30 vol%, some micro/nano-sized pores, with pore size < 0.5 µm, are present on the scaffold surface, but not connectivity with each other, or just partially interconnected. While the $f_{VP}$ is above 40 vol%, microporous structures are obviously present in the scaffolds, their micropore sizes become bigger, and from tens of nanometers to several micrometers, but usually < 5 µm, as shown in Figure 3(D–F). Importantly, in the microporous scaffolds, the micropores are interconnected. Figure 3F shows the microporous scaffolds from resins with 60 vol% porogens, with interconnected micropores. However, the microporous scaffold in Figure 3F appears to be formed by the deposited aggregation of independent nanoparticles,[31] which is very different from the microstructures in Figure 3D and 3E. Furthermore, when $f_{VP}$ is quite high, especially $f_{VP} \geq 60$ vol.%, some macroporous structures will also be over-cured, as shown in Figure S2 and Figure 2B, which is likely because of the enhanced phase separation at higher $f_{VP}$ and thus an increased light scattering of the resins, which will decrease the 3D printing quality.
Cubes with dimensions of 10x10x10 mm, from resins with different \( f_{VP} \), were printed for the measurement of microporosity. Their density and microporosity were calculated by measuring geometric dimensions and by Archimedes law (buoyant force), as shown in Table S2. The microporosity of scaffolds increased with \( f_{VP} \) in the inks. When the \( f_{VP} \) is \( 0–20 \) vol\%, the microporosity is \( 0–5 \)%.
While the \( f_{VP} \) is up to \( 30 \) vol\%, the microporosity of scaffolds is only \( 7 \)% (less than \( 10 \)%). These data indicate that occluded pores maybe still fill with octanol and alcohol due to the non-interconnected micropores while the \( f_{VP} \) is below \( 30 \) vol\%. There is a turning point at the \( f_{VP} = 30 \)%, for the microporosity increase with increasing \( f_{VP} \) in the inks, as shown in Table S2. While the \( f_{VP} \) is \( 40–60 \) vol\%, the micropores become interconnected and the solvent of octanol and alcohol can be washed out of the scaffold, and the microporosity reached \( 20–40 \)%.

The SEM images in Figure 3 also reflect the variation of the scaffolds' microporosity in Table S2.

### 2.2 Cell proliferation

Microporosity can boost specific surface areas to offer more protein adsorption sites, and generate capillary force for improving the attachment and proliferation of bone-related cells on the scaffolds surface.\(^4,33\) However, some studies also reported that higher microporosity was unfavorable for the proliferation of bone cells.\(^4,34,35\) Therefore, influence of microporosity of the printed scaffolds on cell attachment and proliferation was systematically studied \textit{in vitro}. The 3D printed bowl-shaped microporous scaffolds with flat surfaces were used as supporting surfaces for cultivating MC3T3-E1 preosteoblast cells in 24-well microplates. As shown in Figure 5, MC3T3-E1 cells adhered and grew onto the surface in the culture period.
FIGURE 5  Fluorescent microscope images of F-actin staining (Alexa Fluor 488 phalloidin) and cell nuclei staining (NucBlue Live ReadyProbes) of MC3T3-E1 preosteoblast cells after 1, 7, and 15 days’ expansion, on 3D printed bowl-shaped microporous scaffolds (bPG0, bPG20, bPG30, bPG40, and bPG50), fabricated from DEGDA with different porogen $f_{vp}$ of 0 vol%, 20 vol%, 30 vol%, 40 vol%, and 50 vol%, respectively.
FIGURE 6 Viability tests for MC3T3-E1 preosteoblast cells growth on the 3D printed bowl-shaped porous scaffolds over a 15-day period, assessed by Microplate AlamarBlue® assay. The porous scaffolds (bPG0, bPG10, bPG20, bPG30, bPG40, bPG50, and bPG60) are prepared with photopolymerization inks: DEGDA with different porogen f_{VP} of 0 vol%, 10 vol%, 20 vol%, 30 vol%, 40 vol%, 50 vol% and 60 vol%, respectively. * and ** indicate $P < .05$ and $P < .01$, respectively.

from day 1 to day 15. The fluorescent images in Figure 5(A-E) show that the adherent cells spread on the surfaces after seeding for 1 day. The cell attachment efficiency on bPG0, bPG40, and bPG50 are higher than those on bPG20 and bPG30, as shown in Figure 5(A-E). After 7 and 15 days' culture, cells on bPG0, bPG40 and bPG 50 have a higher expansion rate than on bPG20 and bPG30, as shown in Figure 5(F-O). Clearly, cells on bPG40 and bPG 50 also have better spreading and morphologies than on bPG20 (Figure S3).

Figure 6 shows the results of cell viability tests carried out on the 3D printed bowl-shaped microporous scaffolds. At day 1, cell viability on bPG0 has no significant difference from bPG 40 and bPG50, but is significantly higher than bPG10, bPG20, and bPG30. This may be due to the higher attachment of cells on PG0, PG40, and PG50 scaffold, as compared to bPG10, bPG20, and bPG30. During the cultivation from 4 days to 15 days, cells viability on both bPG40 and bPG50 scaffolds showed significantly higher ($P < .01$) than on other scaffolds. It was proved that scaffolds, with more than 20% microporosity and micropore size from hundreds to thousands of nanometers, can promote in vitro expansion of preosteoblast cells. However, these scaffolds (bPG10, bPG20, and bPG30) have very poor performance, on which cell viability is significantly lower than bPG0, bPG40, and bPG 50. This may result from the low porosity ($< 7\%$), non-interconnected micropores, small pore size ($< 500$ nm) of these scaffolds (Figure 3A-C), and long term toxicity from octanol/alcohol slowly leaching out from these occluded micropores. These results also indicated that low porosity and non-interconnected micropores with small size are detrimental for cell growth. These results show that there is a lower threshold micro-porosity between 7\% and 20\%, which is required for attachment and proliferation of MC3T3-E1 preosteoblast cells (Figure 4). This is because microporosity can increase specific surface areas for more protein adsorption, and generate capillary force to improve the attachment of bone-related cells on the scaffolds surface. However many papers also reported that higher microporosity was not always beneficial for the proliferation of bone-related cells. Microporous scaffold bPG60, with a higher microporosity of 36\%, also has a very poor cell attachment and proliferation, on which cell viability is significantly lower than bPG0, bPG40, and bPG 50 after 7–15 days' culture. It is because cells have difficulty adhering onto the surface of the PG60 scaffold due to the structure of nanoparticle-like aggregation (Figure 3F), which is different from the network structure seen in Figure 3D and 3E.

In bone scaffolds, a highly porous structure with dual macro- and micro- porosity may promote oxygen, nutrient and waste exchange, and are favorable for the cell attachment, migration, bone growth, vascularization. Therefore, in order to study effects of dual macro-and micro-porous structures on in vitro culture of preosteoblast
cells, 3D printed honeycomb-structured scaffolds with micropores (Figure S2) were used to proliferate MC3T3-E1 preosteoblast cells. Figure S4 shows the spreading and growth of MC3T3-E1 cells on the honeycomb-structured scaffolds after 15 days’ expansion. Cells have the highest proliferation on the hPG40 scaffold. Cells grow well in the holes on the scaffolds of hPG0 and hPG40, and moderate in the holes on the hPG30 scaffold. However, cells can grow out from the holes onto the top surface of the hPG40 scaffold, but not onto the top surface of the hPG0 and hPG30 scaffold. Cell viability tests also were carried out on the 3D printed honeycomb-structured microporous scaffolds, as shown in Figure S5. Generally, the results are similar to those on the bowl-shaped microporous scaffolds. The cells viability on the hPG40 and hPG50 scaffolds has no significant difference after 15 days’ expansion, but is significantly higher than on other scaffolds. The cell proliferation rates on the honeycomb-structured scaffolds is higher than on the bowl-shaped scaffolds, especially on hPG0 and hPG30. It indicates that the dual macro-/micro-porous structures are beneficial for the proliferation of MC3T3-E1 preosteoblast cells. The results from Figure 6 and Figure S5 show that there is a performance range of microporosity on MC3T3-E1 cell adhesion and proliferation, as shown in Figure 4.

3 | CONCLUSION

Microporous scaffolds have been successfully printed on a DLP printer with a stable mixture of porogens and photopolymerizing resin. Porogen concentration has great effects on the microporosity and pore size of the scaffolds. Both microporosity and microspore size of the scaffolds increase with the porogen concentration in the ink. The microporosity and microspore size of scaffolds mediated the in vitro expansion of preosteoblast cells. At a lower microporosity between 7% and 20 % or a higher microporosity in the range of 30%-35%, preosteoblast cells showed a poor attachment and proliferation. We found that the best microporosity range for the printed scaffold is between 20% and 30%, where cell proliferation rate was significantly higher than on solid scaffolds, together with a much better spreading and morphologies. Scaffolds with lower microporosity (< 7%) seems “toxic” for cells growth the scaffolds with a higher microporosity (> 35%) showed a poor cell attachment and is unfavorable for the expansion of bone cells. We also showed that the scaffolds with dual macro-/micro-porous structures were beneficial for the proliferation of MC3T3-E1 preosteoblast cells, as compared to the mono porous scaffold with either only the macro-porous or the microporous structures.

4 | EXPERIMENTAL SECTION

4.1 | Materials

Di(ethylene glycol) diacrylate (DEGDA), Bisacylphosphine oxides (BAPOs), and 1-Octanol were purchased from Sigma-Aldrich. Ethanol was obtained from Green Tropic Products Pte. Ltd. The mouse calvarial pre-osteoblast cells (MC3T3-E1 Subclone 4, ATCC CRL-2593) and trypsin were purchased from the American Type Culture Collection (ATCC, USA). Minimum Essential Medium α, fetal bovine serum, penicillin-streptomycin, alamarBlue, Alexa Fluor 488 phalloidin and NucBlue Live ReadyProbes Reagent were purchased from ThermoFisher Scientific.

4.2 | 3D printed microporous scaffolds

Vat photopolymerization ink, composed of a monomer of DEGDA, photo-initiator of BAPOs and a ternary porogen mixture, was prepared for 3D printing of microporous scaffolds. The ternary porogen mixture consisted of 50 vol% 1-Octanol, 30 vol% ethanol and 20 vol% pure water, which was homogenized by a disperser (Model: IKA T18 digital) at 6000 rpm for 5 minutes. In detail, BAPOs/DEGDA solutions was firstly prepared by dissolving 1% (w/v) photo-initiator of BAPOs into 50 mL plastictubes with the DEGDA resin (50, 45, 40, 35, 30, 25, and 20 mL, respectively). Then 0, 5, 10, 15, 20, 25 and 30 mL of the porogen mixture was added into the above 50 mL plastic tubes with BAPOs/DEGDA, respectively, followed by homogenization at 6000 rpm for 5 minutes. Seven types of the vat photopolymerization inks were obtained, in which the volume fraction \( f_{VP} \) of the porogen mixture is 0%, 10 vol%, 20 vol%, 30 vol%, 40 vol%, 50 vol%, and 60 vol%, respectively.

Fifty milliliters of these inks were poured into the transparent photopolymer trough, and printed by vat photopolymerization under a DLP 3D printer (model: ANYCUBIC PHOTON 5.5), with designed STL files (Figure 1). After 3D printing, the scaffolds, with four designed shapes (cube, bowl shape, honeycomb, and diamond lattice), were rinsed twice with ethanol, followed by an irradiation for 10 minutes under UV light in the LZC-ORG Photoreactor. The scaffolds were incubated in ethanol overnight to remove the residues of 1-Octanol and DEGDA. After dried, seven types of scaffolds (named as PG0, PG10, PG20, PG30, PG40, PG50, and PG60), from the inks with different \( f_{VP} \) (0 vol%, 10 vol%, 20 vol%, 30 vol%, 40 vol%, 50 vol%, and 60 vol%, respectively), were obtained, in which bowl-shaped scaffolds were named as...
bPG0, bPG10, bPG20, bPG30, bPG40, bPG50, and bPG60, respectively; honeycomb-structured scaffolds were named as hPG0, hPG10, hPG20, hPG30, hPG40, hPG50, and hPG60, respectively.

Bowl-shaped scaffolds were coated with a thin layer of gold (20–30 nm), sputtered using a JEOL JFC-1200 Fine Coater. Their surface morphology was imaged using a JEOL FESEM JSM6700F scanning electron microscope (SEM), operated at 5 kV. Photographs of scaffolds were taken by an iPhone 8 camera.

4.3 Microporosity measurement of microporous scaffolds

The 3D printed cubes were used to measure the microporosity of scaffolds from the inks with different porogen fVP. Their microporosity was calculated with their density, which was measured by two methods: geometric dimensions, and Archimedes law (Buoyant force).[39]

4.3.1 Geometric dimensions

Firstly, dimensions of cubes were measured with a digital micrometer (calliper), and their volume (Vb) was calculated. Their corresponding weight (mb) of cubes was measured in air by an electric balance. The bulk density (ρb), also known as the apparent density, can be calculated by:

$$\rho_b = \frac{m_b}{V_b}$$  \hspace{0.5cm} (1)

4.3.2 Archimedes law (Buoyant force)

Archimedes principles are applied to calculate the density when sample weight is measured sequentially in the air and liquid. Therefore, weight of cubes was firstly measured sequentially in the air (mair) and water (minwater). The bulk density can be calculated by:

$$\rho_b = \rho_w \frac{m_{\text{inair}}}{m_{\text{inair}} - m_{\text{inwater}}}$$  \hspace{0.5cm} (2)

where ρw is the water density.

The measured density of solid cubes (PG0), from inks without porogens, was defined the solid (true) density (ρs) of the material of polymerized DEGDA. Then the microporosity of microporous scaffolds can be expressed as:

$$\text{Microporosity} = \left(1 - \frac{\rho_b}{\rho_s}\right) \times 100\%$$  \hspace{0.5cm} (3)

4.4 Cell culture

The MC3T3-E1 preosteoblast cells were cultured in a T75 tissue-culture flask (Nunc) in Minimum Essential Medium α, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and maintained for one week in a 37°C 5% CO2 humidified incubator (Thermo Scientific). The cultured preosteoblast cells were used in cell experiments on scaffolds.

4.5 Cell proliferation assay

The 3D printed bowl and honeycomb structured scaffolds (their designed outer diameter: 15.6 mm) were sterilized in an autoclave at 121°C for 15 minutes, and placed into individual wells (well diameter: 15.6 mm) of 24-well cell culture plates. One milliliter of culture medium was added to each scaffold and allowed to incubate for 1 hour. After incubation, MC3T3-E1 cells were seeded on the scaffolds at a density of 1.0 × 10^4 cells/well. The cell culture plates were then placed in a 5% CO2 humidified incubator at 37°C with medium change every alternate day.

4.6 Cell viability tests

The cell viability was measured on day 1, 4, 7, 10, and 15 by alamarBlue assay. At each time point, after the culture media was removed, 1 mL fresh culture medium and 100 µl alamarBlue was added into each well, followed by incubation in a 5% CO2 humidified incubator at 37°C for 4 hours. After the incubation time, four 100 µL aliquots of the alamarBlue conditioned medium from each well were transferred into a 96-well plate (Greiner Bio-One). The alamarBlue conditioned medium without cells were used as negative controls. And cells grown on 24-well cell culture plate, made of tissue culture polystyrene (TCPS), were used as positive control.

4.7 Fluorescent imaging of cells on the 3D printed scaffolds

After being cultured for 1, 7 and 15 days, medium was removed from the wells, and cells were washed twice with pre-warmed PBS (pH 7.4). Then cells were fixed with 400 µL of 4% paraformaldehyde in PBS buffer (pH 6.9)
for 20 minutes at room temperature, followed by washing twice with PBS (pH 7.4). These cells were permeabilized in 400 µL of 0.1% Triton X-100 in PBS for 5 minutes, and then washed twice with PBS. Fixed cells on the scaffolds in the wells were stained for 30 minutes at room temperature, with 400 µL of 300 nM Alexa Fluor 488 phalloidin (a high-affinity F-actin probe) solution in PBS, and 1 drop of NucBlue Live ReadyProbes Reagent (nuclei stain). After washing twice with PBS, the cell-scaffold constructs were imaged using a confocal laser scanning microscope (Leica, TCS SP5X) equipped with a 10x magnification dry lens, and excitation wavelengths of 405 and 488 nm were used.

### 4.8 Statistical analyses

Experiments were performed at least in triplicates, with data values reported as mean and standard deviation. Statistical significance of differences was calculated by a one way analysis of variance (ANOVA), followed by a post-hoc Tukey HSD (Honestly Significant Difference) Test. Significance was accepted at two levels: *P < .05 and **P < .01.

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### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

### DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION
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