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Effect of selective oxidation of bacterial cellulose on degradability in phosphate buffer solution and their affinity for epidermal cell attachment

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Abstract

Bacterial cellulose (BC) has a very promising application in biomedical engineering due to its three dimensional nano-network and good biocompatibility. However, it is difficult for BC to degrade in vivo without cellulase, which has limited its potential application. In this work, oxidized bacterial cellulose (OBC) was prepared according to selective oxidation with NO_2 gas. The structure and micromorphology of OBC were characterized by FTIR, XRD, and SEM respectively. The results showed that the oxidation did not break the crystal structure and the crystallinity of BC. OBC still kept the 3D nano-fibrils network, while the diameter of each fiber in the nano-fibrils network of OBC became wider. When immersed in PBS, OBC degraded gradually. The mass loss rate and degradation rate of OBC were much higher than those of BC after degradation for 60 days. Degradation occurred from surface to inside and the oxidized part of the network favored the process. Results of cell-adhesion and proliferation studies also revealed that OBC had excellent cellular affinity as that of BC.

Keywords: Bacterial Cellulose, Oxidization, Degradability, cellular affinity

1. Introduction

As a polymeric material with nanofiber-network structure, BC has various perfect properties such as high water holding capacity, high crystallinity, high tensile

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strength, good biocompatibility as well as in situ moldability and low production cost [1-3], which has made it to be one of the most promising tissue engineering scaffold materials. At present, BC has a good application in wound covering, brain membrane, skin of tissue engineering, renovating artificial blood vessels and cornea etc [4-6]. However, the degradability of BC in human body has become one of the key factors that constrain its potential application in tissue engineering, since there is no cellulase in body fluids.

Oxidization of cellulose is an effective method to modify the chemical structure in order to improve its degradability in vivo for biomedical applications [7]. Cellulose has already been oxidized by different oxidant such as TEMPO-NaClO-NaBr [8-10], NaIO4 [11, 12], NO_2 [13, 14], $HNO_3/H_3PO_4/NaNO_2$ [15] through which the primary alcohol groups at C6 were selectively oxidized into carboxyl during these oxidation process. Oxidized cellulose can be used as Surgicel to stop bleeding [16]. Besides, the oral adsorbent which is gotten from 2, 3-dialdehyde cellulose compounds can also function well in treating Chronic Renal Failure [17]. Recently, 2, 3-dialdehyde bacterial cellulose (DABC) oxidized by periodate was prepared [18], which made its degradation rate in PBS was faster than that of BC, but the crystallinity and mechanical strength were much lower. Nitrogen dioxide (NO_2) with excellent selectivity is considered as a more suitable oxidant for cellulose [19] among the oxidants as secondary reactions were almost avoided. We have used NO_2 to oxidize bacterial cellulose in gaseous environment for different time and got OBC with carboxyl groups at C6 [20]. The oxidation did not affect the crystal structure of BC, but changed the morphology of its network with wider diameter. Results showed that the oxidation reaction was controllable for regulating the degree of degradation of BC. However, structural changes, aggregative state during degradation, and cellular affinity of OBC have not been studied yet. Besides, degradation rate of OBC according to glucose concentration in PBS was not detected either.

In this work, we focused on further discussing and analyzing the degradation of oxidized bacterial cellulose. The degradation performance in vitro including changes in degradation rate, chemical and crystal structural and micromorphology changes of

OBC and BC were characterized and compared. Simultaneously, degradability of OBC was analyzed and degradation mechanism was then proposed. The cellular affinity of OBC was also evaluated and compared with that of BC.

2. Experiments

2.1. Preparation of OBC

Hainan Yida Food Co, Ltd supplied the BC membranes. As a pre-treatment, BC membranes were immersed in 10% NaOH solution for 30min at 80°C to remove the bacterial cell debris, then thoroughly washed to neutral by de-ionized water. NO_2 was produced by adding a suitable amount of Cu to superfluous HNO_3 (65 wt %) solution. NO_2 reacted with purified BC (15 mm in diameter and 1 mm in thickness) for 3, 6, 9 and 12 days following the method last used.

2.2 Degradability in vitro

Degradation of BC and OBC was tested *in vitro in* phosphate buffered saline solution (PBS, PH=7.4) at 37°C. Films of BC and OBC were cut into circular slices with a diameter of 15mm, and then immersed in PBS in centrifuge tubes. The samples were taken out and washed by de-ionized water separately after degradation for 3, 6, 9, 12, 15, 18, 21, 24, and 30 days, then freeze-dried and weighed. The original mass of each sample was designated as m_0 while the mass after degradation was characterized as m_1. Mass loss rate is the ratio of reduced mass and original mass, which was calculated by the Equation (1).

Ultraviolet spectrophotometer (Unico, UV-2100) was used to test the glucose content as degradation product. Its absorbency at 470 nm has a liner relationship with the concentration of glucose solution. Degradation rate is the ratio of actual glucose mass and original mass which was measured to analyze degradation degree of OBC. Concentration of glucose was noted as C and volume of degradation solution was designed as V. Degradation rate were calculated by the Equation (2) [21].

$$Mass\ loss = \frac{m_1 - m_0}{m_0} \times 100\% \qquad (1)$$

$$Degradation\ rate = \frac{C \times V}{m_0} \times 100\% \qquad (2)$$

Special software Spss 13 was also used to statistically analyze the data of mass loss and concentration of glucose related to degradation rate. The data between three groups were analyzed with variance. All data were represented by average± standard deviation ($\bar{x} \pm$sd).

2.3 Cellular affinity test

Three groups of materials (named group a: blank control, group b: OBC, group c: BC) were cut into discs with a diameter of 15 mm and heat-sealed after package with plastic zip-lock bags. Samples were then sterilized under 18.40kGy with radiation of Co60. Experiments of cell cultivation were performed in compliance with the relevant laws and institutional guidelines and approved by No. 1 Hospital affiliated to General Hospital of the Chinese People's Liberation Army.

Newborn fetal rat was executed and immersed in 75% ethanol for 3 min. After that, subsequent operations were all under aseptic conditions. Torso skin was sheared off from the body of the rat. Then torso skin was cut into pieces of 0.5 cm^2 and immersed in a mixture solution of 0.25% trypsin and 0.02% EDTA for 16h at 4^oC in refrigerator. Then epidermic was removed from the dermis and put into centrifugal tube containing dulbecco's modified eagle medium (DMEM). Epidermal cells formed cell suspension due to blowing with straw. The original number of epidermal cells was microscopically counted and centrifugation treatment at the speed of 1000 r/min for 5min was followed subsequently. DMEM solution containing 10% tire bovine serum was added to the suspension, adjusting the density of cells into 1×10^4 /mL. The suspension was then inoculated in 24-hole training board coated with 1 ml mouse-tail collagen per hole in advance.

Three groups of materials were placed in holes respectively after suspension's sedimentation for 1 h. They were then incubated in hatch box under the condition of 5% CO_2 at 37°C. Culture solution was renewed at the time of 4 and 7 days

respectively. After 1, 4, 7, 10 days, cells cultivated with different materials were taken out and observed under optical microscope, then the cell numbers were microscopically counted and cell viability was tested through MTT method.

2.4. Characterization

The microstructure of BC and OBC was observed by scanning electron microscopy (SEM, Apollo 300, and 10 KV). All of the samples were freeze-dried and coated with a thin layer of gold in a sputter coater in advance.

Chemical structure of BC and OBC before and after degradation for different time was tested by Fourier Transform Infrared Spectroscopy (FTIR, NICOLET 750) with a range of frequency from 4000 cm^{-1} to 450 cm^{-1} and a resolution of 4 cm^{-1}.

X-ray diffractometry (XRD, D/MAX-RB, 20 kV, 40 mA) with Cu kα radiation (λ=0.154nm) was used to examine the crystal structure of the samples. The range of diffraction angles (2θ) was 10°-40°.

3. Results and Discussion

3.1 Mass loss and degradation rate

The mass loss rate and degradation rate are often used to assess the degradation of biomaterials in vitro [22]. When immersed in PBS, both BC and OBC began to degrade from surface in, degraded debris and fragments dropped from original films of BC and OBC, leading to mass loss of original materials. The degradation product of cellulose is glucose, so as for oxidized cellulose. Therefore, glucose content represents their biodegradability in a way. Standard curve of glucose concentration showed the content match the absorbance at 470 nm wavelength in linear relationship well. The glucose concentrations of different degradation stage were measured accurately, and the degradation rates were calculated according to Equation (2).

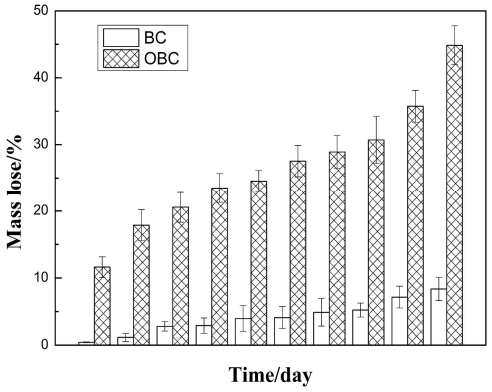


Fig.1 Mass loss rate of BC and OBC

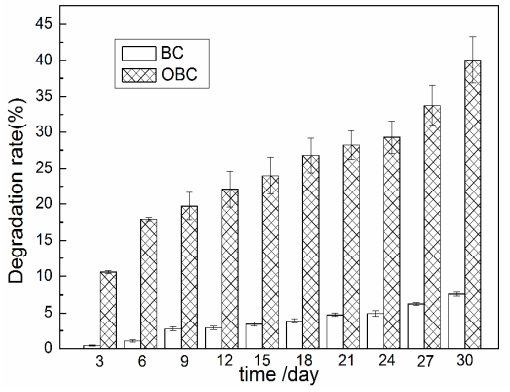


Fig.2 Degradation rate of BC and OBC

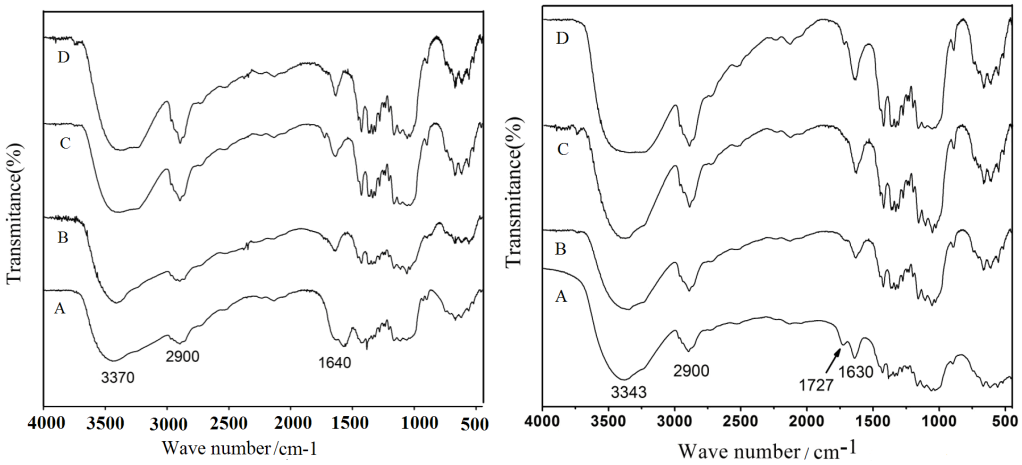
Fig.1 shows the mass loss of BC and OBC. Just as expected, the mass loss rate of OBC was much higher than that of BC obviously. For BC, the mass loss rate was less than 10% (wt) even after 60 days in PBS solution (Fig.1), which was in consistence with the results reported by Peng [20] and Li [18] . For OBC, however, it even rose up to 45% as time extends. For DABC as reported by Li[18], the mass loss rate was about 80% after 1000h (about 41.5 days). Apparently, the mass loss rate of OBC in Fig.1 was lower than that of 2, 3-dialdehyde bacterial cellulose (DABC), this is mainly because oxidation by NO_2 is much milder [19] than that of periodate and degradation accompanied with oxidation was slight. Moreover, OBC with 45% mass loss after degradation *in vitro* for 60 days still maintained its initial shape and partial mechanical properties, implying more or less that oxidation occurred in the BC membrane was a uniform selective one. Besides, the high crystalline degree and less crystal defects of OBC may also helped to interpret its slow degradation speed in comparison with electrospun plant cellulose oxidized by NO_2 [23].

The existence of glucose in PBS solution suggests that the C-O-C bond become weakened and cracked after degradation, which contributed to the production of small molecular of glucose as well as the degradation of BC and OBC. Degradation rate of BC and OBC (Fig.2) kept the same trend with mass loss rate as time passed. The degradation rate of BC was less than 8% after 60 days, while for OBC, it came up to 40% (Fig.2), indicating that degradation of OBC in PBS solution was greatly sped up and OBC was easier to degrade in PBS solution with its characteristic carboxyl groups at C6 due to the selective oxidization.

Another significant phenomenon is that during early times of degradation, the mass loss rate was always higher than degradation rate for BC as well as for OBC. This is because mass loss was calculated according mass before and after degradation, while degradation rate was achieved through glucose concentration in PBS. When materials began to degrade, small fragments such as fiber bundles left materials, causing mass loss. However, these bundles do not decomposed into glucose completely at once, leading to relatively lower degradation rate than mass loss rate. Overall, there is no big difference between mass loss and degradation rate, since most of the fiber bundles finally degraded into glucose after falling off. Therefore, we are sure that BC and OBC have degraded in PBS solution and OBC possess a higher degradation rate.

3.2 Structural changes of BC and OBC after degradation

The hydroxyl groups at the position C6 of cellulose could convert to carboxyl groups when selectively oxidized by NO_2. Fig.3 shows the chemical structure of OBC together with BC before and after degradation for different time.



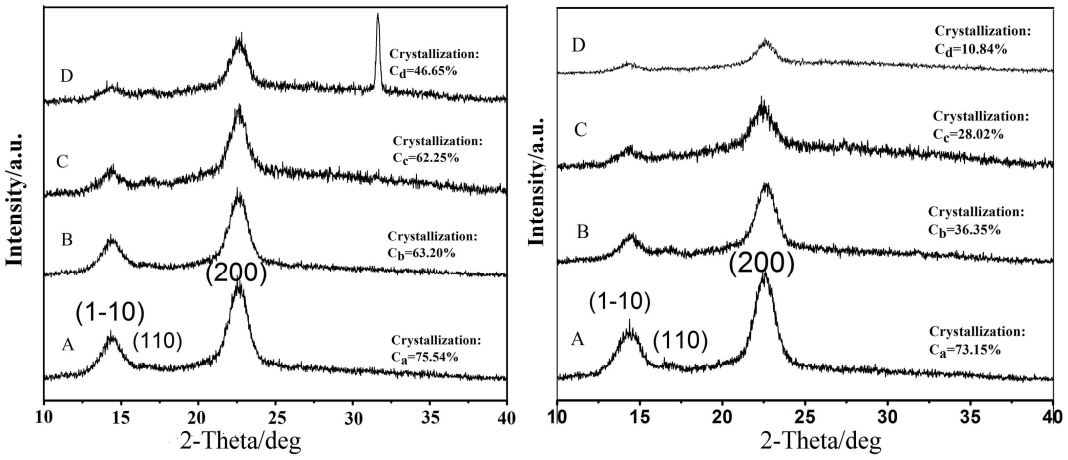
(a) (b)

Fig.3 FTIR spectra of (a)BC and (b)OBC degraded in PBS respectively for (A) 0 day, (B) 12 days, (C) 30 days, (D) 60 days.

For original BC, there are three absorption peaks at 3370, 2900, and 1060 cm^{-1} corresponding to the stretching vibrations of the group ν(–OH), ν(-CH_2) and ν

(C-O) respectively [24, 25] (Fig.3a curve A). Compared with FTIR curve of original BC, new absorption peaks appeared at 1727cm^{-1} assigned as C=O stretching vibration of carboxyl groups after oxidation, indicating the existence of the oxidation product — carboxyl cellulose. Meanwhile, the intensity of absorption peaks near 1320-1210 cm^{-1} associated with the C-O groups decreased obviously, further proving the formation of OBC (Fig.3b curve A). After degradation in PBS for different time, the peaks of hydroxyl groups of BC were all obviously strengthened (Fig.3a curve B, C, D and Fig.3b curve B, C, D), while the typically characteristic peak of OBC appeared at 1727cm^{-1} corresponding to C=O stretching vibration disappeared (Fig.4b curve D). That means more hydroxyl groups formed due to the broken of C-O-C bond and degradation occurred at the position of C6.

3.3 Crystal structure comparison of BC and OBC after degradation



(a) (b)

Fig.4. XRD patterns of (a)BC and (b)OBC immersed in PBS for (A)0 day, (B)12 day, (C)30 days, (D)60 days.

Fig.4 shows the XRD patterns of BC and OBC degraded for different times. For original BC (Fig.4a curve A), three peaks are located at diffraction angles of 14.6°,16.5°and 22.5° respectively corresponding to the primary diffraction of the crystal plane (1-10), (110) and (200), which shows the structure of well-defined cellulose I crystal [26-29]. The diffraction peaks of OBC according to Fig.4b curve A are almost the same as those of original BC, implying that OBC still retained the crystal

structure of cellulose I and the oxidation had little impact on the crystal structure as well as their crystallinity [30].

When immersed in PBS solution for different time, crystallinity of both BC and OBC decreased，the crystallinity of BC decreases from 75.54% to 46.65% as time extends from 0 day to 60 days (Fig.4a), while for OBC, it ranged from 73.15% to 10.84% (Fig.4b). This is because with the presence of carboxyl groups and higher activity, a long-term degradation of OBC enabled water molecules to permeate into inner crystalline regions, leads to a loose arrangement of molecular chains and expanded amorphous regions[31, 32]. At the same time, degradation in amorphous regions was under way and all above helps to drop crystallinity down. Besides, the intensity of diffraction peaks mentioned above weakened as time passed, especially for OBC. Peaks at crystal plane (1-10) of OBC nearly disappeared after immersion for 30 days and 60days (Fig.4b curve D). Obviously, crystallinity of OBC decreased more sharply compared with BC, indicating that OBC was much easier to degrade in PBS solution because of the active action of carboxyl groups at C6 position. The similar phenomenon was also reported by Li [18] and Calvini [33].

3.4 Surface morphology

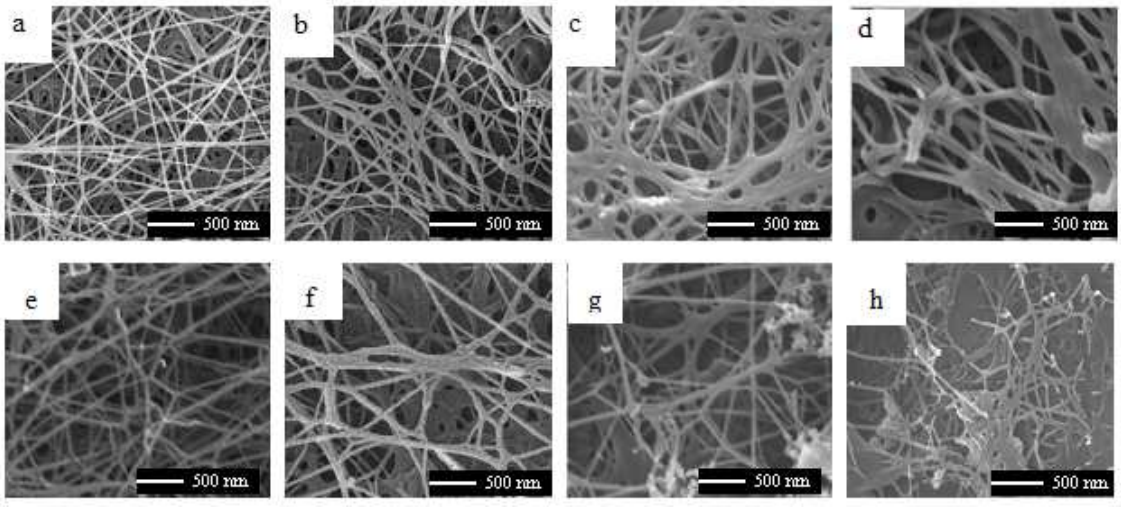


Fig.5. SEM of BC and OBC before (a, b, c, d) and after (e, f, g, h, corresponding to a, b, c, d respectively) degradation for 60days (a)BC, (b) OBC oxidized for 3 days, (c) 6 days, (d) 12 days.

Fig.5 showed the SEM images of BC and OBC before and after degradation for 60 days. BC had a dense 3-dimension network structure of the nanofibrils, which was almost retained after oxidation. Meanwhile, the nanofibrils of OBC appeared to aggregate or fuse into bundles gradually after oxidization for different time (Fig.5b, c, d). In addition, a few fibrils began to be fragmentized by the oxidation process (Fig.5d). That is to say, oxidation was accompanied by a light degree of deterioration visible on oxidized fibers, which was similar to the phenomenon described by Kim [15] and Kumar [34].

After degradation in PBS solution for 60 days, BC maintained the original nano-fibrous networks with a few fibrils ruptured (Fig.5e). While for OBC oxidized for 3 days, more fibrils ruptured and its network became loose (Fig.5f). The 3D nano-fibrous network of OBC oxidized for 6 days partly disappeared, and many fibrils fracture and fell into pieces (Fig.5g) because of the accelerated degradation of OBC. More OBC fibers eroded and the nano-fibrous network was almost collapsed after degradation for 60 days for OBC oxidized for 12 days (Fig.5h), which wass in consistent with its high mass loss rate and degradation rate of 40% above. Obviously, the oxidization of BC by NO_2 sped up the degradation of OBC, which could gradually destruct nano-fibrous network of OBC at a desirable degradation rate by controlling time.

3.5 Discussion and analysis on degradation mechanism

It is well known that BC degrades very slowly without cellulase, however，oxidization can help to improve its degradability, which has been proved by results above. When immersed in PBS, both BC and OBC would illimitably swell from surface in, and the crystallinity and the binding force of hydrogen bonds within and between molecular chains would decline [35], leading to the fracture of hydrogen bonds at the position of C2, C3 and C6, which could induce the formation of new hydrogen bonds with water molecule. For both BC and OBC, swelling first began from amorphous regions and surface of crystalline regions during degradation. That is to say, degradation occurred from amorphous regions and surface of crystalline regions,

then moved to inner crystalline regions when increasing the time. Breakage of structure turned from crystalline regions to amorphous ones, leading to the decrease of crystallinity and causing the wideness and excursion of hydroxyl groups peaks (Fig.3). This process contributed to the further swelling and degradation of cellulose macromolecular chain as a long-term swelling enabled water molecules to permeate into inner regions and turned it to be a looser arrangement of molecular chains with expanded amorphous regions [36]. Then the bond C-O-C between monomers broke and degraded parts dropped from surface, leading to mass loss. After that, the original inner parts turned out to be surface and expose to PBS to repeat the process. This regular pattern of degradation is in line with that of 2, 3-dialdehyde bacterial cellulose reported by Kim [12] and Calvini [33].

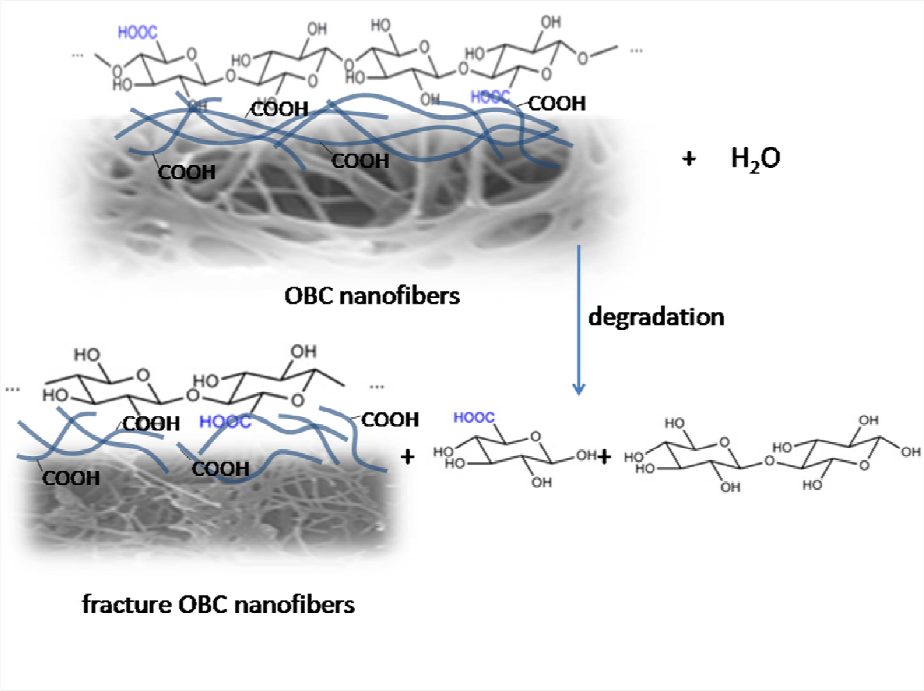


Fig.6 Degradation and breaking of OBC molecule chains

What made OBC degrade faster than BC is that after oxidation, carboxyl groups of OBC at C6 possess higher energy as well as better activity. Meanwhile carboxyl groups are easier to rotate around bond of C5-C6 than hydroxyl group, which makes the bond between monomers of OBC more liable to fracture than BC. The degradation process of OBC is shown in Fig.6. Molecule chains of OBC first break into short chains with different degrees of polymerization, then these shorter chains

finally degraded into glucose. This phenomenon was close to the reported value for the periodate oxidation modified on BC [20, 33]. Besides, OBC with better hydrophilia due to carboxyl group at C6 enables OBC to absorb more water molecules, which will also contribute to its hydrolysis. Furthermore, as mentioned in 3.3, the crystallinity of BC was 75.54% while the crystallinity of OBC was 73.15%. With the presence of carboxyl groups and higher activity, OBC enabled water molecules to permeate into inner crystalline regions, leads to a loose arrangement of molecular chains and expanded amorphous regions which caused a faster degradation. All above helps to drop crystallinity down. The disappearance of C=O peaks (Fig.4.b) proved that the molecular chains break into terminal groups at the position of carboxyl group when they degraded in PBS solution, which lead to reduce even complete vanishing of peaks of carboxyl groups.

Crystalline region contributes greatly to the strength of cellulose[37] and mechanical properties of cellulose often deteriorated by secondary oxidation process[25]. Therefore, it is necessary to investigate the mechanical properties and other physical properties of OBC oxidized by NO_2, which is an ongoing study.

3.6 Cells cultivation

Direct culture method was adopted to evaluate and compare the cellular affinity of OBC with BC. The epidermal cells of fetal rat were co-cultivated with three groups of materials (a: blank control, b: OBC and c: BC) for different time. After seeding epidermal cells on these samples, the cell proliferations status and cytomorphology after cultivation for 1, 4, 7, and 10 days are shown in Fig.8, respectively.

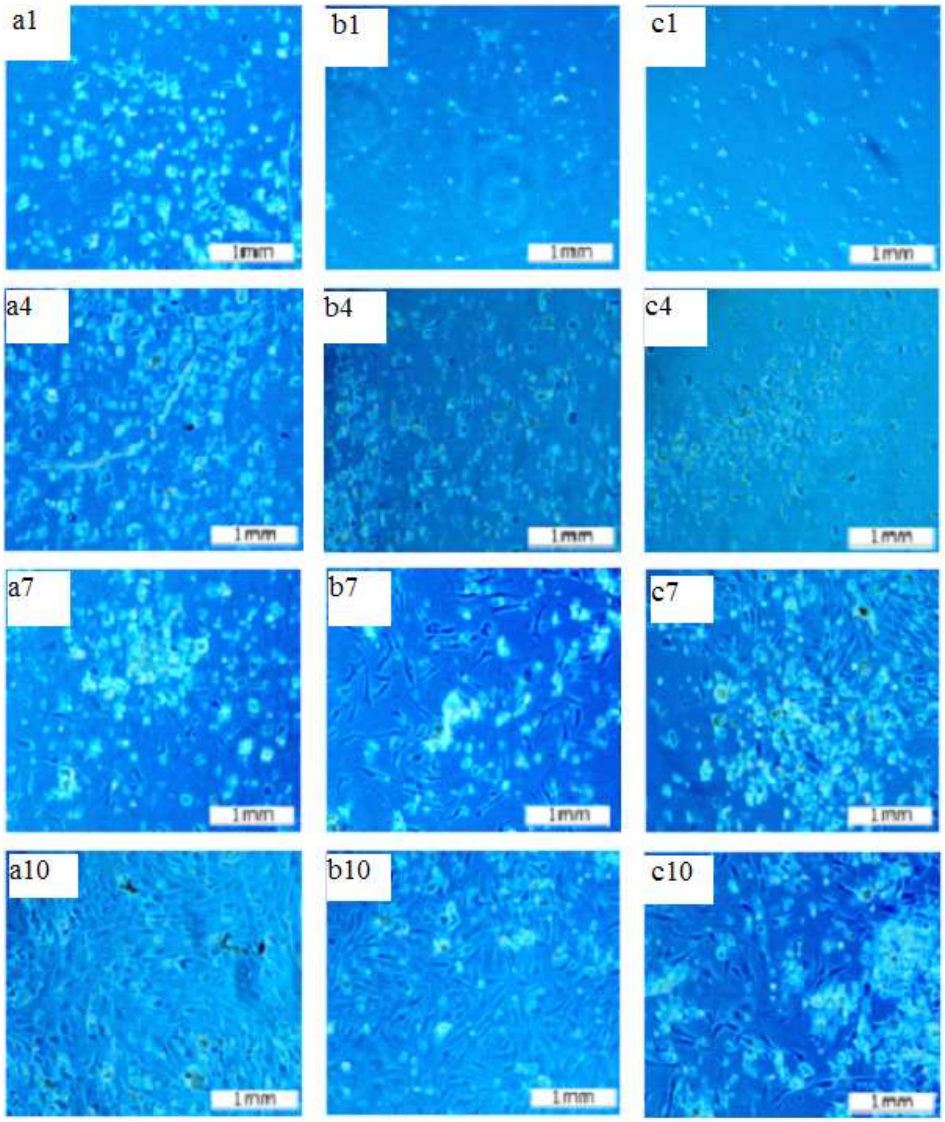
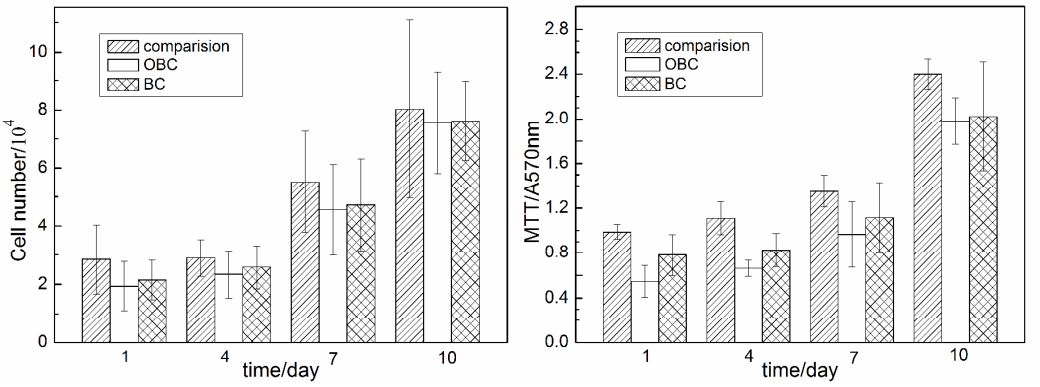


Fig.7 Optical photos of proliferation status (cytomorphology) of epidemal cells after cultivation with different samples (a: blank control, b: OBC and c: BC): a1, b1 and c1 for 1 day; a4, b4 and c4 for 4 days; a7, b7 and c7 for 7 days; a10, b10 and c10 for 10 days.

Obviously, non-transparent small cells with an orbicular shape observed under optical microscope are epidermal cells while others are fibroblast ones. On the whole, cells amount rose continuously as time extended. As cultivated for 1day, a small number of cells distributed in DMEM solution dispersedly (Fig.7). The cells number of group was more than that of group b and group c. After 4 days, more colonies of epidermal cells in three groups appeared together with several fibroblast cells. Cells gathered and overlapped mutually. Cells on the edge grew outside with a form of

single layer, contributing to the extension of colonies (Fig.7). Orbicular epidermal cells and spindly fibroblasts proliferated rapidly when it came to 7 days. Epidermal cells merged into small areas. At the same time, the missdistance among three groups decreased. After 10 days, fibroblast cells proliferated abundantly, especially for group a, in which fibroblast cells almost filled the whole area. For group b and group c, more epidermal cells grew and their numbers increased apparently (Fig.7). The total of epidermal cells and fibroblast ones in each group became almost the same finally, suggesting that BC and OBC both support cell proliferation.

3.7 Cellular affinity evaluation



(a) (b)

Fig.8 Cellular affinity of OBC compared with blank control and BC (a) epidermal cell numbers during the proliferation, (b) MTT of cells cultivated with different materials.

Fig.8a shows specific number of epidermal cells during proliferation when cultivated in vitro. Just the same as the results observed in Fig.8, epidermal cells number grew slowly during early days and then became fast. The numbers of epidermal cells cultivated with three groups of materials all slightly rose after one-day proliferation and cells of group is a little more than that of b and c. After 4 days, epidermal cells numbers in three groups all increase slightly, in which group a is still the most. When it comes to 7 days, epidermal cells proliferate faster than before and differences among three groups minished ($P<0.05$). When cultivated for 10 days, distinction among cells numbers of three groups almost significantly disappeared ($P < 0.05$). On the whole, cell growth of three groups share the consistent trend without great differences, suggesting that BC and OBC have little impact on cell proliferation

and they both support cell proliferation with good cellular affinity.

The MTT data describe the relative viability of cells including epidermal cells as well as fibroblast cells growing on the surface of different materials. The data are comparable since the same numbers of cells were added to samples. Consequence of MTT colorimetric estimation is shown in Fig.9b. MTT values of group a kept the most all along the cultivation. MTT values of cells cultivated for 4 day were almost the same with that of cells cultivated for 1day. When 7 days passed, data increased a little. While for 10 days, MTT values rose up to a high level resulted from the proliferation of both epidermal cells and fibroblast cells. These materials hardly affect proliferation of epidermal cells. As result of MTT colorimetric estimation is in line with cell growth number to same extent, we can arrive at the conclusion that OBC and BC support cell proliferation with good biocompatibility, which extends their potential application in vivo as implanted materials.

4. Conclusions

OBC oxidized by NO_2 in aphotic condition keeps the original 3D nano-fibrous network crystal structure of BC with relatively wider diameter and it is prone to degrade in PBS solution more easily according to mass loss and degradation rate compared with BC. For OBC, the mass loss rate went up to 45% after 60 days while it was only approximately 10% for BC. FTIR spetra of OBC immersed in PBS for different time also suggest that the degradation procedure of OBC first occurs from amorphous regions and surface of crystalline regions, then moves to inner crystalline regions. Carboxyl groups with higher energy and better activity are easier to rotate around bond of C5-C6 than hydroxyl group, help the bond between monomers of OBC more liable to break than BC. Oxidation has little effect on the crystal structure of bacterial cellulose. After degraded in PBS solution, Crystallinity of OBC decreases sharply, indicating that OBC is much easier to degrade in PBS solution and further proved the degradation mechanism. According to SEM micrographs, OBC oxidized for different time degraded at varying degrees with fibrils fragmentized more or less. When cultivated with epidermal cells of fetal rat, OBC showed a good cellular affinity,

contributing to a wider application in tissue engineering. Consequently, the selective oxidation by nitrogen dioxide has been proven to be a practicable method for modification of BC's degradability without weakening its biocompatibility and crystal structure.

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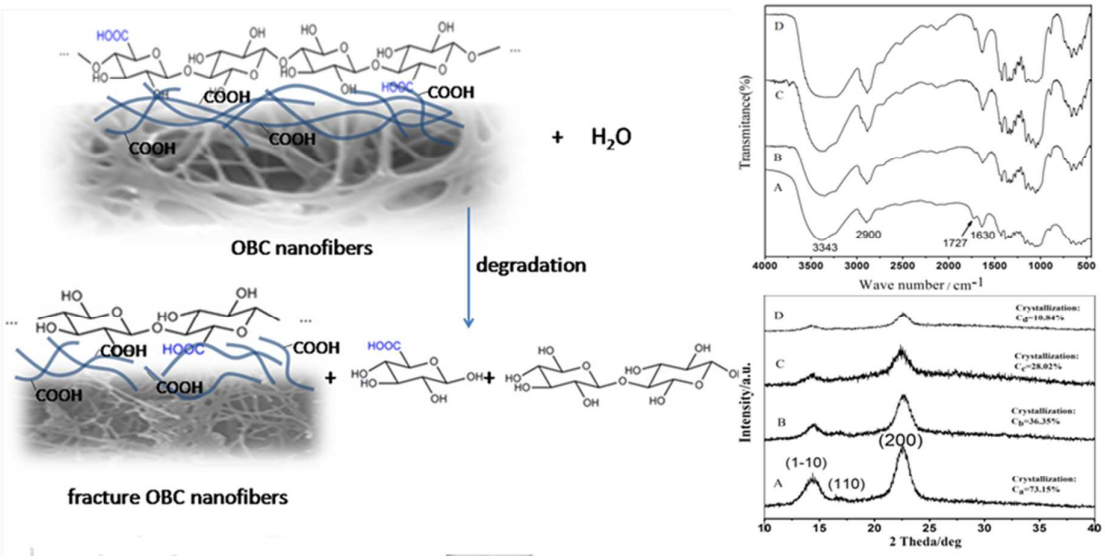
Graphical abstract



The structure and micromorphology of oxidized bacterial cellulose (OBC) selective oxidized with NO_2 gas were characterized. The mass loss rate and degradation rate of OBC were much higher than those of BC after degradation for 60 days. The oxidation did not break the crystal structure and the crystallinity of BC. OBC still kept the 3D nano-fibrils network, while the diameter of each fiber in the nano-fibrils network of OBC became wider. When immersed in PBS, OBC degraded gradually. Degradation occurred from surface to inside and the oxidized part of the network favored the process. Results of cell-adhesion and proliferation studies also revealed that OBC had excellent cellular affinity as that of BC.

Effect of selective oxidation of bacterial cellulose on degradability in phosphate buffer solution and their affinity for epidermal cell attachment

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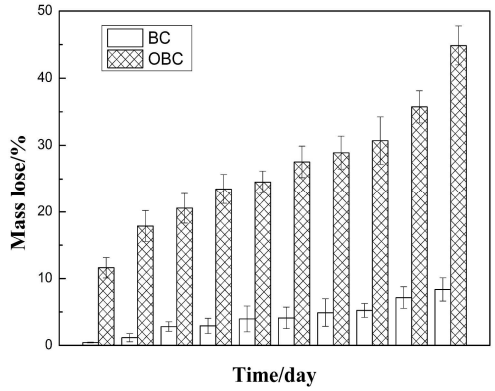

Fig.1 Mass loss rate of BC and OBC

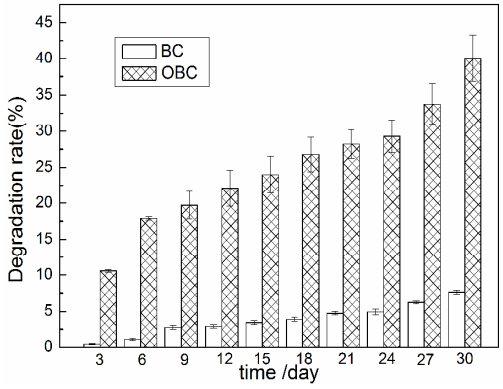

Fig.2 Degradation rate of BC and OBC

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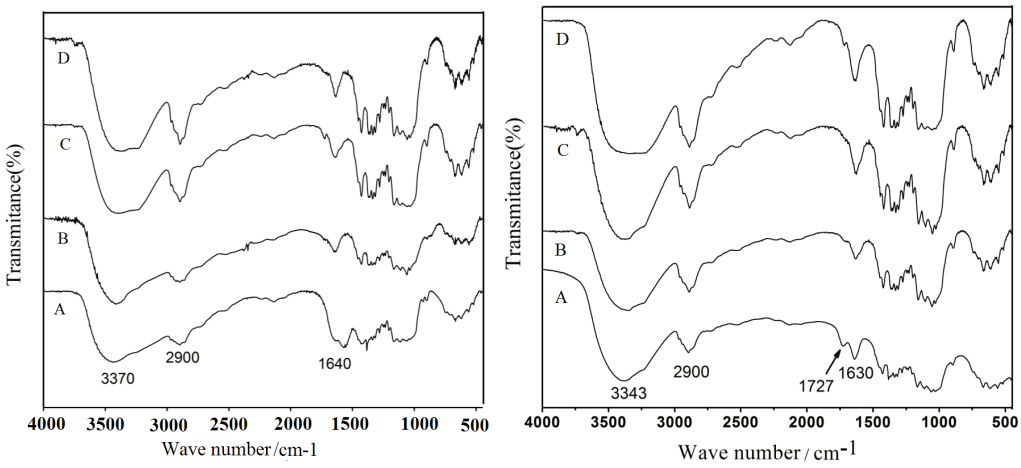
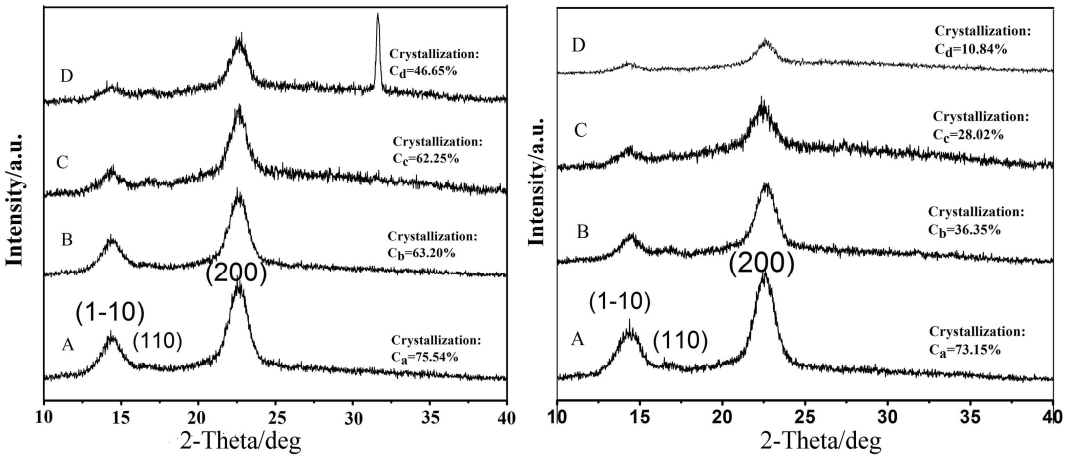


(a) (b)

Fig.3 FTIR spectra of (a)BC and (b)OBC degraded in PBS respectively for (A) 0 day, (B) 12 days, (C) 30 days, (D) 60 days.



(a) (b)

Fig.4. XRD patterns of (a)BC and (b)OBC immersed in PBS for (A)0 day, (B)12 day, (C)30 days, (D)60 days.

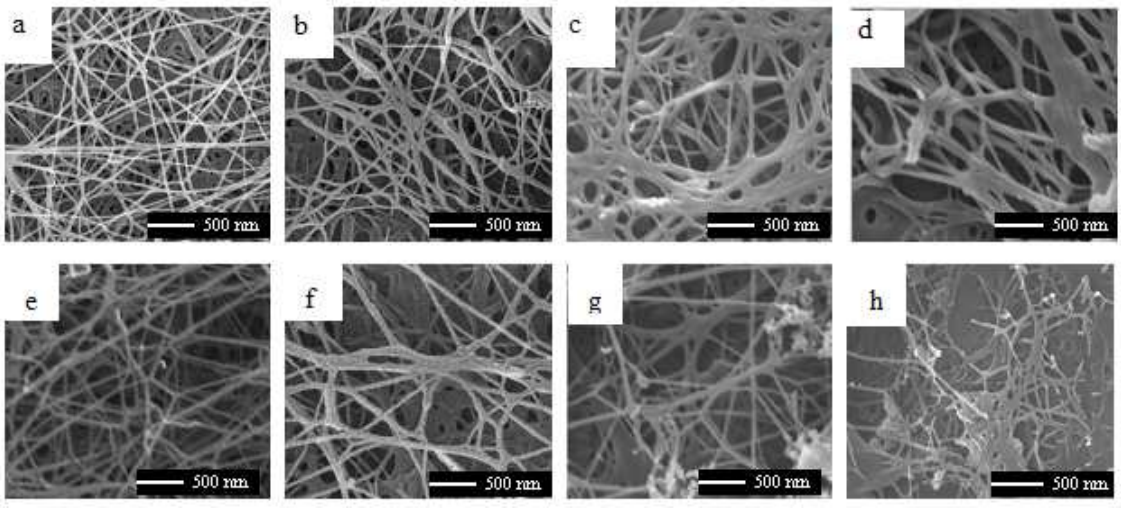


Fig.5. SEM of BC and OBC before (a, b, c, d) and after (e, f, g, h, corresponding to a, b, c, d respectively) degradation for 60days (a)BC, (b) OBC oxidized for 3 days, (c) 6 days, (d) 12 days.

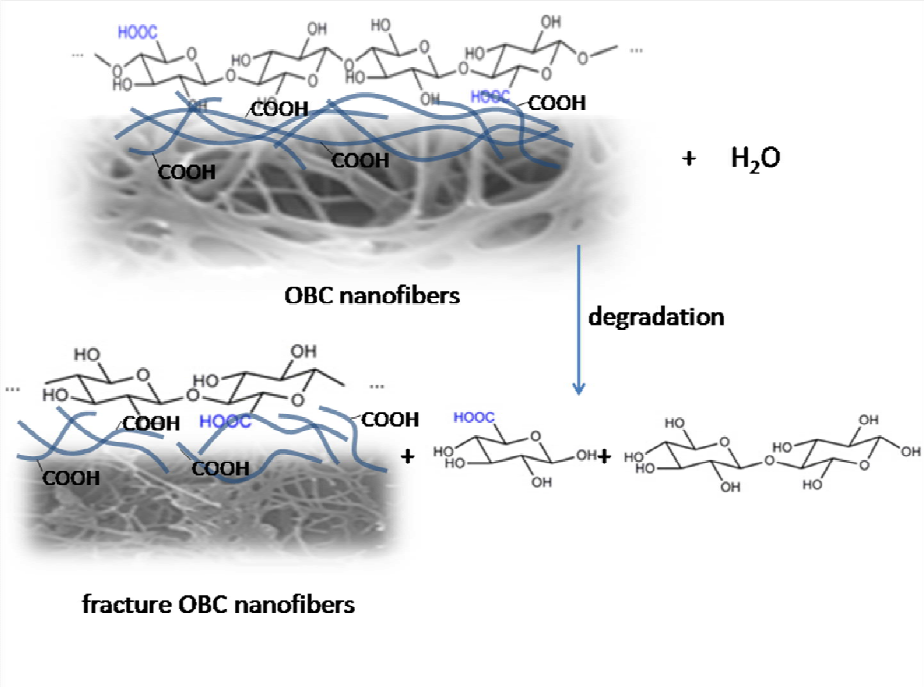


Fig.6 Degradation and breaking of OBC molecule chains

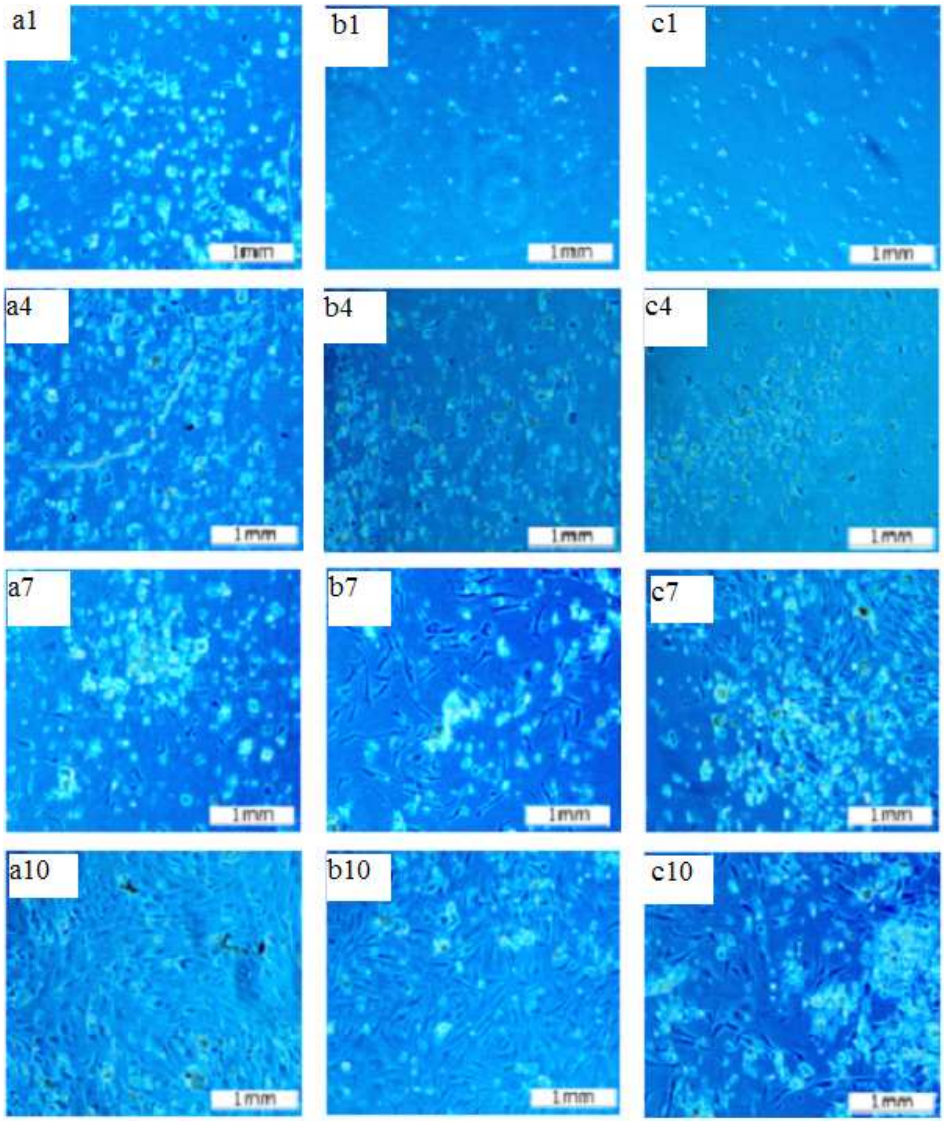
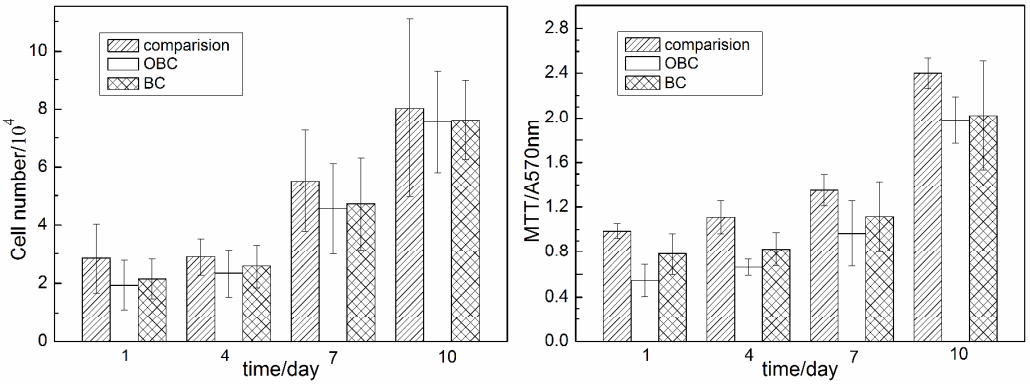


Fig.7 Optical photos of proliferation status (cytomorphology) of epidemal cells after cultivation with different samples (a: blank control, b: OBC and c: BC): a1, b1 and c1 for 1 day; a4, b4 and c4 for 4 days; a7, b7 and c7 for 7 days; a10, b10 and c10 for 10 days.



(a) (b)

Fig.8 Cellular affinity of OBC compared with blank control and BC (a) epidermal cell numbers during the proliferation, (b) MTT of cells cultivated with different materials.