In Vitro Structural Changes of Nano-Bacterial Cellulose immersed in Phosphate Buffer Solution

Yanmei Chen^{1,a}, Tingfei Xi^{2,3,b*}, Yufeng Zheng^{1,c}, Liang Zhou^{4,d}
 and Yizao Wan^{5,e}

¹ College of Engineering, Peking University, Beijing 100871, P.R.China

² Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, P.R.China

³ Shencheng Institute, Peking University, Shencheng 518055, P.R.China

⁴ Haimen Municipal People's Hospital, Haimen 226100, Jiangsu Province, China

⁵ School of Materials Science and Engineering, Tianjin University, Tianjin 300072, P.R.China

^a cym-1223@163.com, ^{b*} xitingfei@tom.com, ^c yfzheng@pku.edu.cn, ^d cannychow@126.com,
^e yzwan@tju.edu.cn

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Abstract. Nano-bacterial cellulose (nBC), secreted by *Acetobacter xylinum*, is expected to have potential applications in tissue engineering. In this paper, the in-vitro degradation performance and the corresponding mechanism of nBC immersed in phosphate buffer solution (PBS) for different time periods was investigated. The pH value variation of solution, material degradation, and the swelling and structural changes of nBC was analysed successively. The results indicate that water molecules attack the exposed nBC fibrils, weakening the bonding strength of inter- and intra-molecular chains and disconnecting partial C-O-C bonds. The disconnection of C-O-C bonds is considered the primary reason for the degradation of nBC large molecular chains after nBC is immersed in PBS. The present work is instructive for controlling the in-vivo degradation performance of nBC acting as bone tissue engineered scaffold materials.

1. Introduction

Tissue engineered scaffold materials, the buttress for osteoblasts adhering and forming chemical bonds with the host tissue, are the fundamental frameworks for tissue engineered bone. At present, in the field of tissue engineered scaffold materials, the main problem is that artificial scaffolds are different to internal ECM in structure and function. Therefore the scaffold materials do not provide a suitable environment for cell proliferation and gene expression, for blood vessel growing in, and for nutritive material transmission. In addition, the degradation rate of the materials does not match the growth rate of new tissue [1]. According to the bionic principle, bionic scaffolds must be structured by nano-fibrils, which has been confirmed by recent research [2]. Moreover, nano-fibril scaffolds are superior to conventional scaffolds for promoting cell differentiation and mineralization [3]. It has been reported that cellular degree of differentiation is related to the diameter of fibril, and cellular rate of differentiation on the nano-fibril is higher than on the micro-fibril [4]. The structure and diameter of nano-fibril and the size and orientation of interspace would interfere with the conglutination and ingrowth of cells [5,6].

Nano-bacterial cellulose (nBC), possessing above particular superiority, has been proved to be modelling material for researchers to study the interaction of scaffold materials with tissue in nano-grade level. Nano-bacterial cellulose (nBC) secreted by *Acetobacter xylinum* is a kind of novel scaffold material used for tissue engineering because of its striking features, such as high mechanical strength and Young's modulus, high crystallinity, biodegradability, porosity, finer web-like network structure, and adjustable aperture [7,8]. Czaja has summarized the application of nano-bacterial cellulose on artificial skin and predicted that nBC would be an important natural material [9]. Previous investigations mainly focused on the applications of nBC for tissue engineered cartilage and blood vessels, but seldom considered the tissue engineered bone [10,11]. Tubular nBC has been co-cultured with endothelial cells in endangium, smooth muscle cells and adventitial fibroblasts, which proves that nano-fibril network structure of nBC is in favour of cellular adhesion, proliferation and ingrowth [12-15].

Nano-bacterial cellulose will contact with water molecules and ions when co-cultured with cells in vitro and implanted in tissues in vivo. But, no research to date has considered the structural transformation of nBC when it is placed in culture solution and tissue fluid. Tissue fluid, a component of extracellular fluid in the cellular interval, is an important place for interchange of substance between cells and blood plasma and is the only pathway for cells contacting with external environment. The inanimate matter in tissue fluid is mainly water and inorganic salt ions. So the degradation of nBC in tissue fluid is unmanageable and non-detectable. Therefore, in vitro degradation is an essential method in studying the degradation performance of scaffold materials. For these reasons, to imitate the in-vivo safety evaluation and investigate the degradation performance of nBC, we soaked nBC into phosphate buffer solution (PBS). The composition of PBS is similar to that of tissue fluid.

The in-vitro degradation performance of nBC after immersion in PBS has been investigated in this paper. The pH value and reducing sugar concentration of solution after immersing flakes of nBC for different time periods are measured successively. The degree of degradation of nBC is calculated by the reducing sugar concentration of the solution. Furthermore, the swelling and structural changes of nBC are analysed in detail. The structural changes of glycosidic bonds in BC molecular chains immersed in saline solution are discussed for the first time.

2. Materials and Methods

2.1 Preparation of nBC. The intact materials of nBC composite are offered by a cooperative union with Tianjin University. The bacterial strain of *Acetobacter xylinum* X-2 was incubated for 7 days in a static culture containing 0.3%(w/w) green tea powder and 5%(w/w) sucrose. After purification and surface modification, nBC with a 3-dimensional (3-D) network was obtained.

2.2 Immersion of BC in PBS. Phosphate buffer solution was prepared by dissolving the following agents in distilled water in order: NaCl, KCl, $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, KH_2PO_4 , CaCl_2 , MgCl_2 . The pH value of PBS was adjusted to 7.40 by a HCl and NaOH solution (Table.1). Then, palaceous nBC weighing of 3.75mg was immersed in 15ml PBS. The longest time of immersion was 33 weeks. The pH value of solution was detected by 654 pH Meter (Metrom, Swiss) each week and the palaceous material was taken out in the 1th, 2rd, 3th, 4th, 5th, 6th, 7th, 8th, 9th, 10th, 11th, 12th, 16th, 20th and 33rd weeks. Each time, four identical pieces of material were simultaneously immersed in four glass bottles filled with PBS, which was used for statistical analysis. The glass bottles containing PBS and nBC were incubated at a constant temperature of $(37.0 \pm 0.2)^\circ\text{C}$.

After immersion in PBS for some time, the materials were cleaned and lyophilized (-50°C) for structure and morphology analysis, and remaining water solution was used to detect the concentration of reducing sugar by ultraviolet spectrophotometer (U-3010, Hitach, Japan). The weight loss ratio and degradation ratio was calculated by the dry weight of residual nBC and concentration of reducing sugar, as follows.

$$\text{Weight loss ratio (\%)} = [(\text{original weight} - \text{dry weight}) / \text{original weight}] * 100\%$$

$$\text{Degradation ratio (\%)} = (\text{weight of reducing sugar} / \text{original weight}) * 100\%$$

Table 1 Concentration and dosage of reagents for PBS

No.	Agents	Concentration(mM)	Dosage(g)
1	NaCl	137.00	8.00
2	KCl	2.70	0.20
3	Na ₂ HPO ₄ ·H ₂ O	10.00	1.56
4	KH ₂ PO ₄	2.00	0.20
5	CaCl ₂	1.00	0.10
6	MgCl ₂	0.50	0.05

2.3 Test methods of structure, morphology and composition of materials. The phase composition of the materials was detected by Japan D/max-γB X-ray diffraction (XRD). The materials were scanned from 10° to 60° with a scan speed of 4°/min. The morphology and microstructure of samples were examined by using a FEI Quanta 200F Environmental Scanning Electron microscope (SEM) with an accelerating voltage of 15kV and electrical current of 10μA. Group structure of the materials was studied by using a Nicolet 750 Fourier Transform Infrared Spectrometer (FTIR). FTIR spectra were recorded in a spectral range of 4000-650cm⁻¹ at a resolution of 8cm⁻¹. Mettler Toledo TGA SDTA 851 Thermogravimetric Analyser was employed to measure the mass changes of materials with heating temperature, the heating temperature from 25°C to 500°C, the rate of temperature increase of 10°C /min, the rate of nitrogen flow of 150 ml/min.

3. Results and Discussion

3.1 The pH value of PBS after immersing nBC. After soaking nBC in PBS for 1 week, the pH value of solution decreased and was below 7.35, as shown in Figure 1, which was caused by the weak acidity of original material during the preparation process. Subsequently, the pH value increased and was approaching to 7.40 by the 6th week. From the 3rd to the 33rd week, the pH value was between 7.35 and 7.40. Since the degradation product of nBC is neutral saccharide, the pH value of solution after soaking nBC does not fluctuate. Vey *et al* [16] studied the degradation mechanism of PLGA co-polymer after immersion in phosphate buffer solution (PBS) without Ca^{2+} and Mg^{2+} . They found that the surfacial structure of the co-polymer would swell and drape successively, and quantified the weight loss and variation of liquid water content of the co-polymer [16].

Figure 1 Changes of the pH value of solution immersing nBC.

3.2 Weight loss ratio and degradation ratio of nBC after immersion in PBS. Employing the method of standard curve with five points, weight loss ratio and degradation ratio of nBC are shown in Figure 2. After being immersed in PBS for some time, nBC micro-fibril will swell infinitely. The binding force of C-O-C bonds in the nBC molecular chains will be weakened and the C-O bonds will break. Subsequently, soluble reducing sugar will form because of the sectional disconnection of molecular chains, which is that nBC degrades slightly. According to the relevant reduction formula, the degradation ratio of nBC is calculated by soluble reducing sugar as 5~9.8%. The reason for the weight loss of nBC mainly is the fragmentation and degradation of nBC. The weight loss ratio of nBC is approximately 5.7% to 10.6%. However weight loss of nBC is primarily caused by fragmentation and degradation of nBC. Therefore weight loss ratio is higher than degradation ratio all the time. With the increase of immersion time, nBC molecular chains will fracture in the region with thin bonding force under the function of water molecules and ions. Ultimately, nBC macromolecules are cut off gradually and transformed into micro-molecular reducing sugars dissolving in PBS. However, without an idiosyncratic catalysing enzyme, the degradation of nBC immersed in PBS is mainly owing to the limitless swelling of nBC fibrils.

Figure 2 Curves of weight loss ratio and degradation ratio of nBC after immersion in PBS.

Figure 3 SEM images of nBC immersed in PBS solution for (a) 0 weeks; (b) 8 weeks; (c) 12 weeks; (d) 33 weeks.

3.3 Morphology of nBC after immersion in PBS. The micro-morphology of nBC in hygrometric state after immersion in PBS for 8, 12 and 33 weeks is shown in Figure 3. Obvious swelling and micro-level fragmentation of nBC fibrils are observed with increasing time, as shown in Figure 3. The reason for the compacting structure was that the bulk material was compacted emphatically for water-wiping. Hydrogen bond of hydroxide group in nBC molecular chain would intensively attract water molecules and positive ions in PBS solution. Hydrogen bonds of the hydroxide group in the amorphous region would break and be replaced by that of water molecules during the dry nBC fibril moisture absorbing. After nBC fibril moisture absorbing, although no changes appear in uniformity of outline, yet the cohesion reduces and the macrostructure intenerates. So infinite swelling emerges in the crystal region, i.e. water molecules will react on the interface between the amorphous and crystal region. Owing to ultrafine nano-structure, nBC possesses higher hygroscopicity, degree of swelling, plasticity and chemical reactive behaviour than advanced plant cellulose. The swelling performance is very important for degradable and macromolecular scaffolds degrading in vivo. The swelling and degradation of material would be limited if the hygroscopic property was poor [17].

3.4 Phase components of nBC after immersion in PBS. The XRD patterns of nBC soaked in PBS are shown in Figure 4. In XRD patterns of nBC, peaks in diffraction angle 2θ of 14.4° and 22.76° are not fully spiculate. Moreover, the majority of crystals orientate in the direction of (200) and the degree of crystallinity is calculated to be 78.34% (from Jade analysis software for XRD patterns). After nBC was soaked in PBS for 12 weeks and 20 weeks, the crystallinity decreases to be about 57.03% and 46.65% respectively.

Figure 4 XRD patterns of nBC immersed in PBS solution for (a) 0 hr; (b) 12 weeks; (c) 20 weeks; (d) 33 weeks.

In the first 20 weeks, swelling primarily occurs on the interface of amorphous regions and secondly occurs in the crystal regions. Crystal structure will transform into a disordered amorphous structure and the bonding strength among molecular chains decreases. After being soaked for 33 weeks, the crystallinity goes down to 26.7% and the peak form of the XRD pattern changes. The lattice plane of (110) nearly disappears and the diffracted intensity of lattice plane of (200) drops. In addition, new α -tricalcium phosphate (α -TCP) comes into being.

From the 20th to 33rd week, water molecules have permeated into the crystal regions and change the original crystal structure, which causes irregular molecular chains and loosens the structure. The bonding strength of hydrogen bonds among molecules and the degree of crystallinity both decrease [18,19]. Hydrogen bonds of hydroxide radical in amorphous region (especially the hydroxide radical connecting the sixth carbon atom) break open constantly, and are re replaced by water molecules. Residual OH^- will combine with H_2PO_4^- and HPO_4^{2-} , and PO_4^{3-} groups produce, and finally a small quantity of α -TCP appears because of PO_4^{3-} combining with Ca^{2+} , which is shown as follows.

3.5 Structural changes of nBC after immersion in PBS. The FTIR spectra of nBC immersed in PBS for 12 and 33 weeks are shown in Figure 5. In the FTIR spectra of nBC, the absorption band at 3345 cm^{-1} is assigned to the hydroxyl group and intermolecular hydrogen bonds [20], the stretching vibrational absorption bands at 2930 and 2853 cm^{-1} is respectively assigned to the asymmetry or epoxide ring C-H bonds and intramolecular hydrogen bonds, and the stretching vibrational absorption band at $1170\text{-}1060 \text{ cm}^{-1}$ is assigned to the C-O bonds.

After nBC is soaked in PBS for 12 weeks, peak shape of FTIR spectra is nearly invariable except that the strength of OH and C-O-C vibrational absorption bands is enhanced and the peaks are broadened slightly. Therefore, we can see that the water molecules have attacked the large molecular chains of nBC. Being in a state of high swelling, bonding strength of intermolecular and intramolecular hydrogen bonds reduces, and moreover, hydrogen bonds of hydroxyl groups connecting with the sixth carbon atom in the amorphous regions will disconnect constantly and then connect with hydroxyl groups of water molecules. The fresh hydroxyl groups connecting with the sixth carbon atom will interact with primary hydroxyl groups connecting with other carbon atoms, which will cause strengthening in the infra-red absorption bands. In addition, because of the reduction of inter- and intra- molecular bonding strength, the radical vibration frequency varies with limits, which is the reason that the spectra peaks of hydroxyl group and C-O bond offset and broaden in FTIR spectra. The spectra peaks of material soaked in PBS for 33 weeks are much wider than that of material soaked in PBS for 12 weeks, which is in conformity with the reduction of crystallinity. Otherwise, the disconnection of partial C-O-C bonds will influence the changes of group structure in the molecular chains of BC.

Figure 5 FTIR spectra of nBC immersed in PBS solution for (a) 0hr; (b) 12 weeks; (c) 33 weeks.

3.6 Thermal effects of nBC after immersion in PBS. The Thermal Gravimetric Analysis (TGA) spectra of nBC immersed in PBS for 12 and 33 weeks are shown in Figure 6. The thermal weight loss value is about 78.9% after original nBC is heated from 250°C to 350°C, which corresponds to the exothermic decomposition peak. In this period, dehydrated cellulose will form after molecular chains of nBC depolymerize, and finally, charcoal and gas will result after glucose residue has decomposed [21,22]. Although the crystallinity decreases after nBC is soaked in PBS for some time, the thermal effects in TGA spectra are not influenced. So the thermal effects of materials after soaking in PBS are similar to those of original materials. After nBC is soaked in PBS for some time, the tendency of the thermal weight loss of materials is identical except that the temperature and extent of thermal weight loss are slightly varied. The thermal weight loss value is 67.5% and 59.8% after nBC was soaked in PBS for 12 weeks and 33 weeks respectively. Moreover, the temperature of thermal weight loss rises a little. In addition, lower thermal weight loss of all materials presented in the initial heating stage, which is because of the weight loss caused by water molecules of physical adsorption and connecting hydrogen bonds.

After immersion in PBS for some time, the nBC will directly contact water molecules, which enables nBC to be in the swelling stage. The hydrogen bonds of hydroxyl groups connecting with the sixth carbon atom will disconnect and the rest of the oxygen bonds of the hydroxyl groups will connect with hydrogen bonds of water molecules, which will form new hydroxyl groups, as shown in reaction formula (2-1). Since the above-mentioned process occurs continuously, the strength of intra-molecular hydrogen bonds will be diminished. Those are the primary reasons for structural changes before nBC is degraded into soluble reducing sugar. In addition, in the special regions of crystal structure transforming into unordered amorphous structure, the bonding strength of partial C-O-C bonds reduces and even the bonds disconnect, which will form small molecular reducing sugars. But without the action of specific enzymes, the C-O-C bonds are difficult to disconnect and also the degree of degradation is lower. The chemical reaction equations of the disconnection of C-O-C bonds are shown as formula (2-2) and (2-3).

Figure 6 TGA spectra of BC immersed in PBS solution for (a) 0hr; (b) 12 weeks; (c) 33 weeks.

The changes of nBC after immersed in PBS are shown in Figure 6. After soaking in PBS for 12 weeks, the nBC fibrils adsorb water molecules and are in a swollen state. Under the action of water molecules, partial crystal regions will be transformed into amorphous regions, and then molecular chains in amorphous regions will be disconnected in succession and be transformed into micro-molecule cellulose, cellobiose, and even glucose (soluble reducing sugar) with increasing time. During the whole process, the structural changes mainly consist of disconnection of network configuration, increasing the strength and vibration frequency of group (-OH, -C-H, -C-O-), which make the crystallinity decrease. In addition, macroscopic changes are the weight loss of the rudimentary materials.

Figure 7 The sketch of degradation progress of BC immersed in PBS solution.

Conclusion

Under the action of water molecules and ions, nBC fibrils will obviously swell. In the swollen state, partial crystal regions of nBC will be transformed into amorphous regions, and the bonding strength of intermolecular and intramolecular chains decreases and the vibrational spectra bands of hydroxyl group, C-H bonds and C-O bonds broaden distinctly because of the disconnection of partial C-O-C bonds, which will form small molecular glucose (soluble reducing sugar) with time. So the disconnection of C-O-C bonds is the primary mechanism of degradation of nBC large molecular chains after nBC is immersed in PBS.

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