Preparation of Di-Butyryl-Chitin Scaffolds by Using Salt Leaching Method for Tissue Engineering and Their Characteristics

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ABSTRACT: Scaffolds are used as support material in treatment of damaged tissues such as cartilage and bone. With the help of scaffolds, damaged tissues can be cured in shorter period with less pain. Chitin is one of the most important scaffold materials curing the damaged tissues while providing a support for related part of the body during healing period. It is biocompatible and biodegradable; however it can not be solved by common solvents leading to the major drawback for this kind of applications. Therefore di-butyril-chitin (DBC), which is a chitin derivative and can be solved easily in solvents like acetone, ethanol, and methanol, is preferred for scaffold production instead of chitin. In this study, DBC scaffolds were produced for orthopedic applications and their structural and mechanical properties such as porosity, elasticity, compressibility, and strength were tested to confirm their suitability for such end-uses. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 2882–2887, 2008

Key words: dibutyryl-chitin (DBC); scaffold; biocompatibility; biomaterials; mechanical properties

INTRODUCTION

One out of 10 patients seeking medical help for knee pain has a significant cartilage injury. Approximately 50% of these have a lesion that would benefit from and is suitable for surgical treatment[1] Joint cartilage shows a very limited capacity for self-repair.[2] Advances in cell-biology and tissue engineering could form a solution for this problem.[3,4]

The *in vitro* culturing of autologous articular chondrocytes and their reimplantation in the injury is already in a further clinical stage.[5] This scaffold-free cartilage regeneration has however several disadvantages.[6] As the *in vitro* grown articular chondrocytes are injected in suspension under a periosteal flap, the function of the replaced tissue is not substituted until the cartilage is fully regrown[7] and the dispersion or orientation is not controlled. Seeding the articular chondrocytes on a three-dimensional scaffold can improve the homogeneous spreading and will support the cells until the cartilage is regrown. The scaffold carrying the cartilage-regenerating cells will bridge the wound after implantation and help to the self-repair to close the gap.[8] When the perfect biomaterial could be developed to make such a scaffold, it would biodegrade just as fast as the cartilage would regrow.[9] As the mechanical properties of this perfect scaffold are as though, compressible and weight-bearing as real articular cartilage, so that the patient would be able to use his knee before the cartilage is fully regrown. There would be an immediate repair, replaced with regeneration over time.

Although many materials have been tested, this perfect biomaterial has not been developed yet. Most of the available materials are based on collagen and other polysaccharides[10] or collagen-derivatives, like Type II collagen-glucosaminoglycan.[11] Other frequently used biomaterials are PGA and PLA,[12] chitosan,[13] gelatin,[14] hyaluronan,[15] and many others. The most frequent problem is cellular dedifferentiation; articular chondrocytes attached to a certain biomaterial loose their ability to create the correct extracellular matrix and regrow cartilage, although this might be helped with growth-factors.[16] Other problems are the inadequate mechanical properties and unsuitable rate of biodegradation of the scaffold. Some biodegradable polymers degrade too fast. For instance, PLA biodegrades within 1 or 2 months that leaves insufficient time for regeneration of the cartilage.[17] The rate of biodegradation of the scaffold should not exceed the regrowth of the tissue. Some scaffolds and hydrogels, like alginate, can not bear enough

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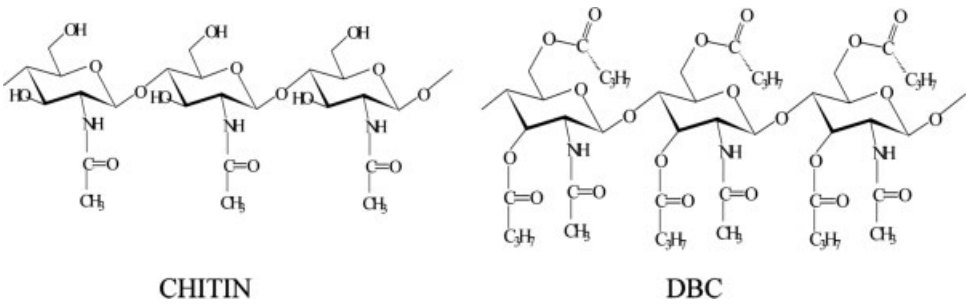

Figure 1 Chemical structure of chitin and dibutyrylchitin.[23]

loads to substitute the function of real cartilage.[18,19] Therefore, new biopolymers and different production methods have been investigated to develop better scaffolds. One example of this kind of biomaterials is di-butyryl-chitin (DBC) which is a derivate of chitin.

Chitin has many features. It is a natural polysaccharide which is a waste material in seafood processing. Chitin and its derivates are partially biodegradable in the presence of human enzymes, and they are nontoxic and beneficial to the human body.[20] When it is used to heal wounded tissues, the healing process is facilitated, allergies and undesirable reactions are not caused, and desired reactions with antiseptic agents occur. However, the main disadvantage of natural chitin application is that it is not soluble in common solvents, and hence causing significant difficulties in chitin processing.[21] On the contrary, DBC is easily soluble in common organic solvents (acetone, ethanol, methanol, etc.) and it can be produced in the form of film or fiber which is essential for scaffold manufacturing. Biological investigations have showed that it has biocompatibility, bacteriostatic characteristics, and sensibility to enzymatic degradation.[22] Structural formulas of chitin and DBC are shown in Figure 1.

In this study, we aimed to develop DBC scaffolds which have suitable structural and mechanical properties such as porosity, compressibility, elasticity, and strength for orthopedic applications.

EXPERIMENTAL

DBC scaffold samples were produced by using salt-leaching method. For this purpose, different chemicals were tried as solvents to find out the most suitable one. Then the effect of salt concentration and size of salt crystals were investigated on the scaffold structure. The optimum ratio of DBC weight to salt weight ($Weight_{DBC}/Weight_{SALT}$) was also determined to obtain the most suitable scaffolds for tissue engineering applications in orthopedics.

After production of the samples, some tests and measurements were carried out to determine their suitability in terms of mechanical behavior and porosity.

Materials

DBC which is a chitin derivative was used as the main material because of its solubility in common solvents as well as biocompatibility and biodegradability.

The synthesis of DBC from shrimp chitin on half-technical scale was carried out at the Institute of Dyes and Organic Products, Zgierz, Poland, in accordance with a Polish patent.[24] The intrinsic viscosity of DBC determined in DMAc solutions at 25°C was 1.70 dL/g, the average molar mass, determined by SEC method coupled with scattering and viscometry, was 132×10^3 g/mol.

Infrared investigations were done using Perkin–Elmer 2000 FTIR instrument. IR spectra have been recorded for both chitin films prepared from solutions of chitin in DMAc/5%LiCl and for DBC films produced using acetone solutions. In IR spectra of DBC samples, no band of absorption was measured at ~ 3450 cm^{-1} due to hydroxyl groups present in IR spectra of chitin samples are visible, but new bands of strong absorption at 1741 cm^{-1}, characteristic for the esters of fatty acids are noticed. Furthermore, bands of stronger absorption appear at around 2900 cm^{-1} corresponding to aliphatic groups $—CH_2—$ and $—CH_3—$, content of which is much higher in DBC than chitin.

Method

The salt-leaching method, which allows the preparation of porous structures with regular porosity, has been used to prepare porous DBC scaffolds employing excess NaCl crystals that can easily be dissolved as a porogen in water easily.[25]

DBC scaffold preparation

During preparation, the DBC solution containing NaCl crystals was pressed into cylindrical plastic molds of 8 mm diameter, and dried at room temperature for 24 h. Then they were put in deionized

TABLE I
The Parameters Used in Sample Production

Test no.	Sample code	Concentration (DBC g/mL)	Solvent type	Salt crystal size (μm)	$Weight_{DBC}/Weight_{SALT}$
Test 1	STE a11	0.10	Acetone	Not sifted	1/10
	STE a12	0.15			
	STE a13	0.20			
	STE a14	0.25			
Test 2	STE e11	0.10	Ethanol		
	STE e12	0.15			
	STE e13	0.20			
	STE e14	0.25			
Test 3	STE m11	0.10	Methanol		
	STE m12	0.15			
	STE m13	0.20			
	STE m14	0.25			
Test 4	STE a21	0.16	Acetone	Not sifted	1/10
	STE a22	0.17			
	STE a23	0.18			
	STE a24	0.19			
Test 5	STE e31	0.21	Ethanol		
	STE e32	0.22			
	STE e33	0.23			
	STE e34	0.24			
Test 6	STE a31	0.17	Acetone	100–200	1/10
	STE a32			200–400	
Test 7	STE e41	0.21	Ethanol	100–200	
	STE e42			200–400	
Test 8	STE a41	0.17	Acetone	200–400	1/5
	STE a42				1/15
	STE a43				1/20
Test 9	STE e51	0.21	Ethanol	200–400	1/5
	STE e52				1/15
	STE e53				1/20

water at room temperature for approximately 24 h to leach out the salt. For each particle size group of crystals, which were chosen as (1) not sifted, (2) 100–200 μm, and (3) 200–400 μm, respectively, in this work, cylindrical macro porous scaffolds were produced with a diameter of 8 mm and a length of 10 mm. To prepare DBC scaffold, the following procedures are carried out to reach the best possible porosity and mechanical properties:

First of all, different solvents were tested for their ability to dissolve DBC, by using acetone, ethanol, or methanol at room temperature. These solvents were dissolving DBC well and homogeneous solutions were obtained. DBC (0.1–0.25 g) was mixed with salt (not sifted particles) of 1/10 of DBC weight, and these mixtures were solved in acetone (Test 1), ethanol (Test 2), and methanol (Test 3), respectively. By this way, four different samples were produced for each solution.

At the second step, the most suitable samples that have enough stiffness and homogeneity were chosen and Test 4 and Test 5 were carried out to determine the optimum concentration by using these selected samples. These samples were produced with four different concentrations, too.

At the third step, the most suitable salt particle size was determined (Test 6 and Test 7). For this purpose, the selected samples with optimum concentration

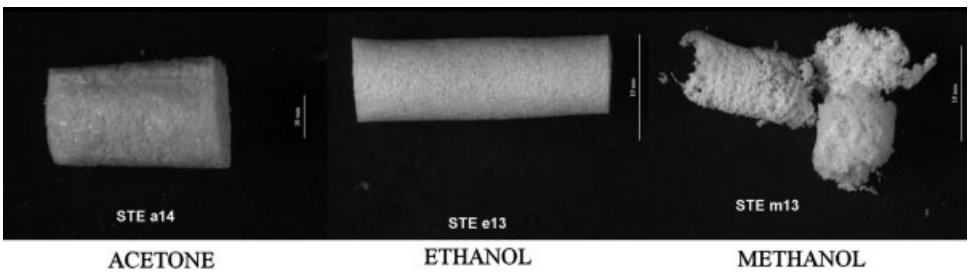

Figure 2 DBC scaffold samples produced by using different solvents.

TABLE II
Test Results of Optimum Samples Produced with Acetone and Ethanol Solvents

Sample name	Porosity (%)	Maximum compression stress (MPa)	Compression modulus (MPa)
STE a22	88	0.294	0.625
STE e31	87	0.397	0.714

were processed with different salt particle sizes at $Weight_{DBC}/Weight_{SALT}$ being 1/10. Thus, the change in mechanical behavior of the samples with different pore sizes could be observed.

Finally, after finding the best concentration and pore size, it was decided to determine the most suitable ratio of DBC weight to salt weight for acetone (Test 8) and ethanol (Test 9) to see the effect of weight ratio on mechanical behavior of the samples. All these steps are explained in Table I.

Compression tests

All samples were compared in terms of their compressibility where the force necessary to compress the sample linearly up to 50% of the sample length to see if the scaffold can carry the weight applied by the body. Then, minimum compression modulus under the linear compression up to 50% of the sample length was determined to see if the scaffold can allow the movement of the body. Finally, maximum porosity values were calculated to see if the scaffold can allow circulation of the blood and other materials like minerals in body mechanism.

In the study, the Instron 3369 tensile tester was used to evaluate compression properties. For the compression measurements, tensile force was converted into a compressive force by a special device designed for this work used. The compression tests are carried out mainly in a vacuumed wet state to simulate the situation in the body. However, STE a32 and STE e42 were tested after soaking in tributyrin for 24 h in a vacuum as well as dry state to see the effect of different conditions.

Calculation of porosity ratio

During the porosity test, the dry weight of the samples was measured first, then immersing them in water and a vacuum was created to ensure the water got into the all pores. Following this, resulting wet weight was determined. The difference between wet and dry mass gives the weight of the water in the pores and knowing the density of water, the volume of the pores could be calculated easily.

The porosity percentage of the samples was calculated by the relation given below:

$$\text{Porosity \% (P\%)} = \frac{z}{t} \times 100$$

where z is the volume of the pores, t is the total volume of the sample.

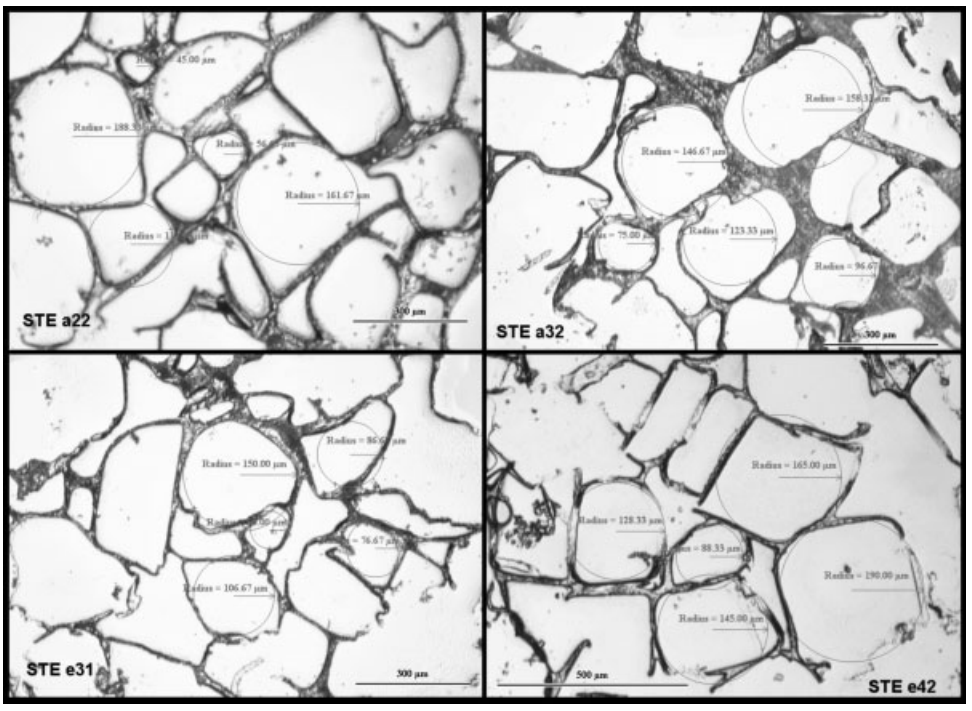

Figure 3 Cross-sectional views of the samples which were produced by using not sifted salt particles and 200–400 μm salt particle sizes, respectively.

TABLE III
The Test Results of the Samples Having Optimum Properties Produced by Test 6 and Test 7

Sample name	Porosity (%)	Maximum compression stress (MPa)		Compression modulus (MPa)	
STE a32	86	Wet state	0.480	Wet state	1.250
		Dry state	0.392	Dry state	2.750
		Soaked in tributyrin	0.343	Soaked in tributyrin	5.750
STE e42	86	Wet state	0.430	Wet state	2.500
		Dry state	0.320	Dry state	5.000
		Soaked in tributyrin	0.285	Soaked in tributyrin	3.375

For a detailed visual analysis of pore size and morphology, Olympus BX51 microscope and Lucia image-analysis system was used.

RESULTS AND DISCUSSION

The results of Test 1, Test 2, and Test 3 showed that DBC dissolves better in ethanol compared to acetone. Interestingly, when DBC scaffolds are left in water for 4 days, some small particles were separated from the DBC treated with acetone leading to the conclusion that DBC was not dissolved in acetone well while the scaffolds treated with ethanol had a more compact structure. On the other hand, the third solvent, methanol, dissolved DBC well, but the DBC treated with methanol and the salt could not be mixed well enough. Therefore, these scaffolds were damaged after salt-leaching as well as low concentrations of this solvent were not stiff and homogeneous enough, so use of methanol as solvent was eliminated for the next steps. The samples produced by using these three solvents are shown in Figure 2 to visualize these effects.

At previous stage (i.e. Test 1 and Test 2), an initial test was conducted to have a general idea regarding porosity, compression force, elastic modulus of the samples as $\sim$ 80%, $\sim$ 0.400 MPa, $\sim$ 0.700–1.200 MPa values were obtained, respectively. Therefore these were taken as the reference values to find out the optimum concentration at the next stage. The results of Test 4 and Test 5 showed that the samples produced at concentration (DBC g/mL) values of 1.7 and 2.1 respectively, had the best mechanical properties and porosity % (samples STE a22, STE e31) where these results can be seen in Table II.

When we analyzed the samples produced by Test 6 and Test 7, where the optimum concentration values and salt of different crystal sizes (100–200 μm or 200–400 μm) are used to determine the suitable crystal size for optimum porosity, some samples having enough strength could be produced by using 200–400 μm salt crystal size while easy cell growth was expected by these samples due to their large pore sizes (Fig. 3). Compression tests were also carried out to these samples in three different forms: dry state, wet state, and soaked in tributyrin for 1 day for comparison. As seen in Table III, the best compressibility was observed in wet state while soaking in tributyrin did not help to improve the compressibility. Dry samples with DBC were damaged during cutting process while they were cut easily in wet state. Samples tested in dry state had higher compression modulus and lower maximum compression stress.

In general, during the compression tests in wet state, where the samples were compressed up to 50% of their original length followed by immersing them in deionized water for 1 day at room temperature, the samples were not able to return to their original length because of broken pore walls except samples of STE a43 and STE e53. The general view regarding broken pore walls after compression test is shown in Figure 4.

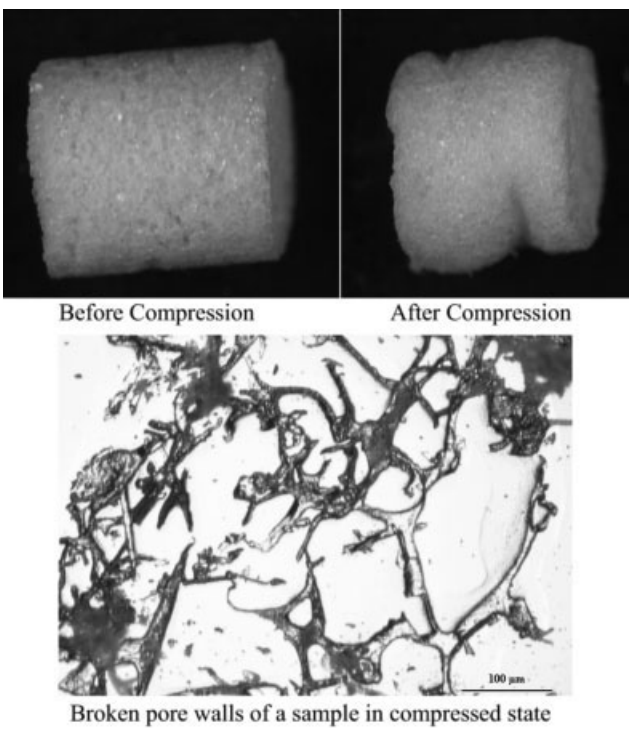

Figure 4 An example view for broken samples after compression.

TABLE IV
The Optimum Properties Obtained by Using $Weight_{DBC}/Weight_{SALT}$ Ratio of 1/20 and 200–400 μm Salt Crystal Size

Sample name	Porosity (%)	Maximum compression stress (MPa)	Compression modulus (MPa)
STE a43	99	0.102	0.400
STE e53	91	0.190	0.585

When we analyzed the effect of the $Weight_{DBC}/Weight_{SALT}$ ratio during Test 8 and Test 9, the optimum results are obtained at ratio of 1/20. Consequently, the aimed results were obtained by samples of STE a43 and STE e53. The mechanical properties and porosity % values of these samples are given in Table IV. The results show that these samples have the necessary minimum compression modulus, strength, and elasticity while having adequate porosity values required for orthopedical applications.

CONCLUSIONS

We developed DBC scaffolds for orthopedic applications by using different solvents, different salt crystal sizes, and different DBC and salt concentrations. The suitability of these scaffolds for such end-use was tested in terms of their compressibility, strength, and porosity. As a result, we found that the best scaffolds can be obtained successfully by using acetone and ethanol, at 1/20 weight DBC/weight salt ratio and 200–400 μm salt crystal size while using methanol gave no satisfying results. Their mechanical and structural properties were determined as compression strength of approximately 0.146 MPa, compression modulus of 0.493 MPa, open pore size of 265 μm on average, and nearly 95% porosity.

In regard to solvent type, both acetone and ethanol gave satisfying results while methanol was found as unsuitable. Between acetone and ethanol, the most suitable one needs to be chosen in further studies depending on the requirements such as sterilization, cell growth, and *in vivo* applications.

In conclusion, the aimed DBC scaffolds were produced by this work successfully. The scaffolds we produced have similar or superior mechanical and structural properties except for compression modulus, which was higher than required compared to the scaffolds of different materials, such as polyurethane, chitosan-alginate, etc. reported by other studies.[26–29] So, the improvement of compression modulus property of this DBC scaffold will be the further study of this on-going research.

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