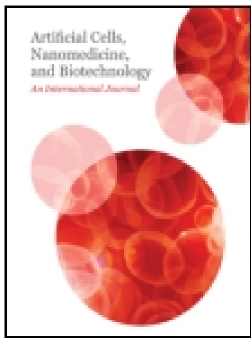

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Influence of RGD grafting on biocompatibility of oxidized cellulose scaffold

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Abstract

Cellulose powder was oxidized by NO_2 gas and the porous scaffold was fabricated by dry pressing. RGD peptide was immobilized on the surface of scaffold by grafting to make a hybrid scaffold. The hybrid scaffold was characterized by SEM and FTIR and its biocompatibility was examined through MTT assay. FTIR results proved oxidization of cellulose and bonding between scaffold surface and RGD. Porous microstructure having suitable size was confirmed by SEM. The results by MTT showed significant increase of viable cells on hybrid scaffold. Porous structure and high biocompatibility were the benefits of scaffold in bone tissue engineering.

Keywords: hybrid scaffold, immobilization, oxidized cellulose, RGD, tissue engineering

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Introduction

Human tissue failures caused by different damages or injuries are the most serious and costly problems in health care and have direct effect on life quality. Some strategies such as autogenic and allogenic tissues (transplantation) have been developed to rectify complications. However, inadequacy of donor organs and lifelong immunosuppression are drawbacks of transplants. Besides, transferred organs cannot function as native one (Puppi et al. 2010).

Bone as a composite material is one of the most important and needful human tissues (Standring 2005). Many bone injuries such as fractures heal well using usual treatments because of its regenerative ability (Puppi et al. 2010). However, surgery strategies became necessary because bone regeneration does not complete in most damages and illnesses (Standring 2005). Also, bone grafts, replacements, or implants are necessary for better cures for wide fractures and osseocongenital deformities (Puppi et al. 2010).

Synthetic and natural materials of different classes including metals, ceramics, polymers, or their composites have been used to fabricate implants. By contrast to metals and ceramics which have no biodegradability and have difficult processability, polymers have high design flexibility due to their structure and composition. Further, degradability in a biological environment can be imparted to polymers through molecular design (Zadegan et al. 2010). Bioactivity and skeletal functions of a bone-replaced material are usually attributed to two key properties: (a) generating an apatite layer on surface in physiological conditions and (b) promoting bone cells attachment, growth, and differentiation (Bartouilh de Taillac et al. 2004). Biocompatibility, biodegradation, and presence of proper functional sites to adapt biodegradation are essential characteristics of a polymer to be implanted properly (Khil et al. 2005). Incapable of cell–polymer interaction in vivo is one of proposed problems for biomedical polymers, which may lead to foreign body reactions such as implant encapsulation, thrombosis, embolization, and aseptic loosening (Hersel et al. 2003).

Considering deficiencies caused by non-biological materials, Tissue Engineering (TE) and stem cells provide promising research fields and may offer innovative viewpoints to treat diseases. In scaffold-based TE strategies, the scaffold serves as template for cells interactions and formation of the extracellular matrix (ECM). Also, it provides structural support for newly formed tissue. Scaffold supports cell colonization, cell migration, cell differentiation and growth, and often controls the developing tissue (Puppi et al. 2010). Porous structure with interconnected porosities, proper functional groups at surface for cell interactions, biodegradability or bioresorbability, needed mechanical properties, nontoxic degraded products, and easy construction are some key characteristics of an ideal scaffold (Verma et al. 2008).

It has been proved that good and strong adhesion of cell to surface restrains apoptosis (Haubner et al. 1996, Stupack

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et al. 2001). Direct contact does not mediate cell response to a biomaterial, but composed layers on biomaterial surface stimulate and mediate cell–biomaterial interactions. Cells can recognize materials through cell-binding peptides in the form of native long chain of ECM proteins or short peptide sequences derived from ECM proteins (Puppi et al. 2010). Biochemical modification can be achieved by a variety of different techniques that exploit either physical adsorption (through Van Der Waals, hydrophobic, or electrostatic forces) or chemical binding involving covalent attachment of the target molecule to a solid surface (Bartouilh de Taillac et al. 2004).

Adsorption of unspecific proteins, improvement of adsorption of specific proteins, and surface modification by immobilizing cell recognition motifs to achieve controlled cell–substrate interaction are some efforts to improve biomaterials (Hersel et al. 2003). Bioactive ligands like peptides and polysaccharides can be adsorbed, grafted covalently onto the surface, or incorporated into the bulk to encourage specific cell adhesion (Puppi et al. 2010). Scientists in various fields have tried to functionalize polymers to achieve special surface cell interactions. In the beginning, they were coated by special proteins like fibronectin, collagen, and laminin which led to some disadvantages. Finding cell recognition motifs as small peptides and immobilizing them on surface were cures for the preceding problems (Hersel et al. 2003).

RGD, a tripeptide consisting of Arginin, Glycine, and Aspartic acid (Arg-Gly-Asp), is more effective and prevalent peptide that improves surface cell adhesion and cell–cell connection. Pierschbacher and Rouslahti detected it in fibronectin as smallest effective peptide on cell adhesion (Pierschbacher and Ruoslahti 1984). RGD, having 346.3 g/mol molecular weight and $C_{12}H_{22}N_6O_6$ molecular formula, was identified to reduce macromolecular ligands to small recognition peptides (Hersel et al. 2003). RGD can be found in von Willebrand factor, osteopoetin, tenascin, and bone sialoprotein. This peptide not only links cell to fibronectin strongly but also makes better cell adhesion (Pfaff 1997). It can be used to address certain cell lines selectively and elicit specific cell responses (Hersel et al. 2003). Also, these peptides can be attached covalently to a polymer through hydroxyl, amino, or carboxyl groups. Functional groups can be introduced onto the surface by blending or co-polymerization (Puppi et al. 2010).

Several synthetic and natural polymers have been studied to form scaffold for bone regeneration. However, inadequate mechanical properties and probable decrease of biological efficacy during formulation compromise use of natural polymers as unique scaffold material contrary to their good biological, economical and environmental aspects (Puppi et al. 2010).

Polysaccharides have several excellent properties such as non-toxicity, water solubility, renewability and stability to pH variations and some weak points like low thermal, mechanical and chemical stability (Puppi et al. 2010). Cellulose and its derivatives, belonging to polysaccharides, are attractive natural biodegradable polymers which have been used in TE. Also, crystalline structure and high molecular weight confer unique properties to it (Zadegan et al. 2010). Crystalline structure of cellulose is arisen by high number of hydrogen bonds from hydroxyl groups which hold cellulose chains together (Verma et al. 2008). Chemical change of cellulose can produce derivatives (cellulosics) having better processability. Cellulosics are biocompatible, recyclable and reproducible, and can be used in biomedical applications. Esterification and etherification at the hydroxyl groups, radical and ionic bonding, acetylation, oxidation, and deoxyhalogenation are chemical modification methods of cellulose (Kamel et al. 2008). Cellulose oxidation causes some structural and crystallinity changes and affects its physical and chemical properties (Verma et al. 2008). Oxidized cellulose (OC) can be made by cellulose reaction with some oxidants such as chlorine, hydrogen peroxide, peracetic acid, chlorine dioxide, nitrogen dioxide, persulfate, permanganate, sulfuric acid-dichromate, hypochloric acid, hypohalite, and periodate. Obtained powder may be consisted of carboxylic, aldehyde or keton functionalities, or hydroxyl groups (Kamel et al. 2008). Although cellulose and its derivatives possess osteoconductive properties, they have no intrinsic osteoinductive capacity and are unable to induce new bone formation in extraosseous sites (Bartouilh de Taillac et al. 2004, Zadegan et al. 2010).

Considering unique characteristics of oxidized cellulose and significant effect of RGD immobilization on cell attachment and division, the present investigation was focused on OC scaffold preparation and immobilizing RGD on its surface. It is expected that the properties of this new material make it promising especially for bone-regenerating applications.

Methods

Oxidation of cellulose

Adequate amount of microcrystalline cellulose ($>90\%$, Merck) was added to a flask. The flask was sealed and NO_2 gas [produced by heating of $PbNO_3$ (>90, Merck) according to the literature (Mallakpour 1992)] was then transferred to it. The flask content was stirred slowly at 38°C for 13 h.

Porous scaffold preparation

A mixture of enough quantity of the oxidized cellulose and polymethylmethaacrylate(PMMA, $>90\%$, Aldrich) as pore generator was molded at 1:1 ratio into a disk-shaped mold having 1 cm diameter and 3 mm thickness and was pressed under 25 MPa. PMMA particle size ranged 300–600 μm. Afterwards, the specimens were put in dichloromethane (DCM, $>90\%$, Merck) solution for 24 h to dissolve and remove the PMMA particles. Finally, the specimens were dried, resulting in porous scaffolds.

Immobilization route of RGD

Protecting of terminal COOH functional groups of RGD

It is necessary to esterificate COOH functional groups of RGD to prevent bonding between amino and carboxyl groups of RGD during immobilization. Adequate amount of freshly distilled thionylchloride ($SOCl_2$, >90, Merck) was dissolved in absolute methanol (>90, Merck) at low temperature, and

1 mmol of RGD (Research Center of Peptide Chemistry, K.N. Toosi University of Technology) was added to solution and stirred for 24 h. The solvent was removed using rotary evaporator.

RGD immobilization

Protected RGD was dissolved in freshly distilled dimethylformamide (DMF, >90, Merck) and 50 mg of TBTU (H-Benzotriazolium,1-[bis(dimethylamino)methylene]-tetrafluoroborate(1-),3-oxide , >90, Merck) was added as peptide coupling reagent. Following it, disk-shaped scaffolds were soaked in the solution for 72 h.

Reactivation of carboxyl groups

RGD-scaffold hybrid was put in 0.1 M NaOH (>90, Merck) for 2 h at ambient temperature and washed several times by double distilled water.

Characterization and parameter calculation

FTIR and SEM analysis

Fourier transform infrared spectroscopy (FTIR) examination (Equinox 55, Bruker) was performed to identify carboxylic groups created on OC over the region 400–4000 cm^{-1}. Also, RGD-scaffold bond was detected by FTIR. Shape, size of porosities, and connection between them were studied by SEM (VEGA II, TESCAN).

Determination of degree of oxidation

Degree of oxidation (*DO*) of cellulose powder has direct relation to the number of carboxyl groups which can be determined by titration. A known amount of oxidized cellulose was dissolved in 0.5 N NaOH and two drops of phenolphthalein (>90, Merck) were added as well. Then, the mixture was titrated using 0.05 N HCl (>90, Merck) to a phenolphthalein end point. The test was repeated thrice. The degree of oxidization and carboxylic acid content (*AC*, w/w%) of OC can be calculated using following equations (Eqs.1 and 2) (Camy et al. 2009):

$$AC = \frac{(V_b - V_a) \times C \times M}{m} \times 100 \quad (1)$$

$$DO = \frac{(V_b - V_a) \times C \times M_{anhydroglucose}}{m - 14(V_b - V_a) \times C} \quad (2)$$

Where V_b is HCl volume which was used to titrate solution without OC (l), V_a is HCl volume which was used to titrate solution having OC (l), C is concentration of HCl solution (mol/l), M is molecular weight of COOH group (g/mol) and m is used OC weight (g). Also, $M_{anhydroglucose}$ is the molar mass of one anhydroglucose unit of the cellulose (162.15 g/mol), and 14 is the difference between the molecular weight of a glucuronic acid unit and an anhydroglucose unit.

Measurement of porosity percent of scaffold

Scaffold was soaked in double distilled water for 24 h. Weights of specimen before and after water taking were measured. Porosity percent (*P*) of scaffold can be calculated using Eq.3:

$$P = \frac{W - D}{D} \times 100 \quad (3)$$

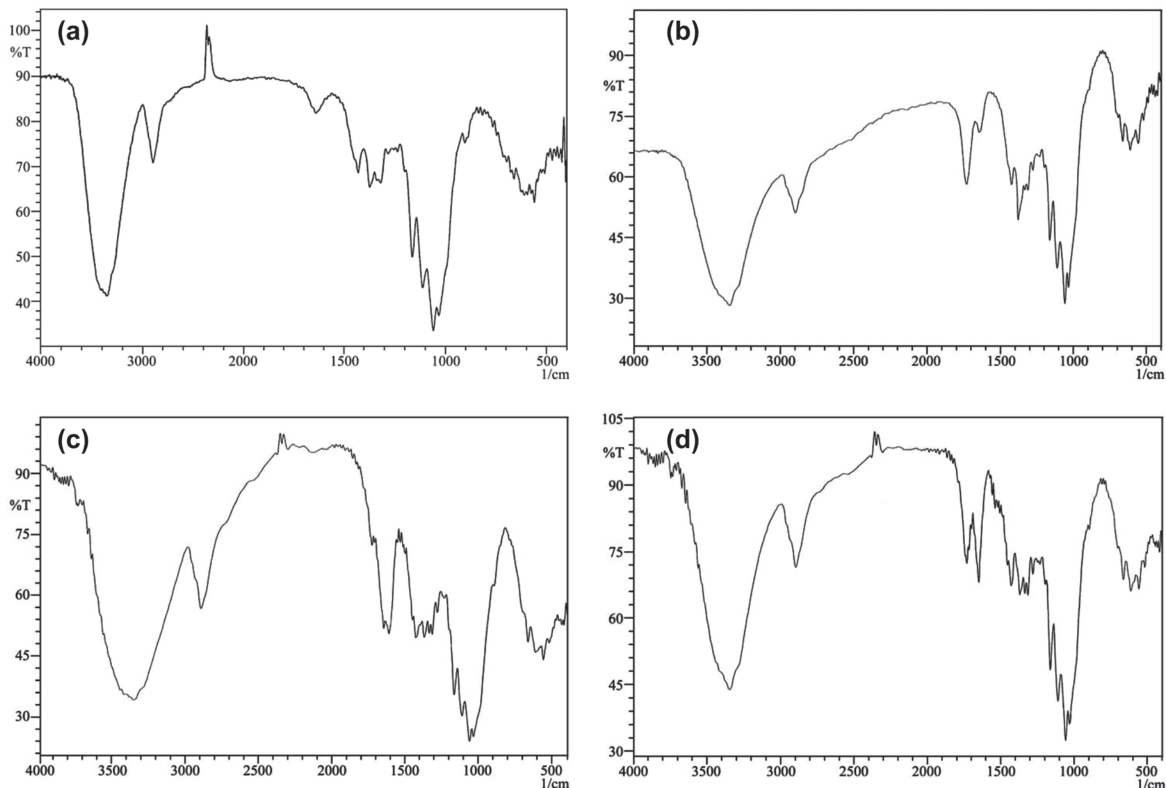


Figure 1. FTIR spectra of (a) cellulose (b) oxidized cellulose (c) OC-RGD hybrid before reactivation of RGD (d) OC-RGD hybrid after reactivation of RGD.

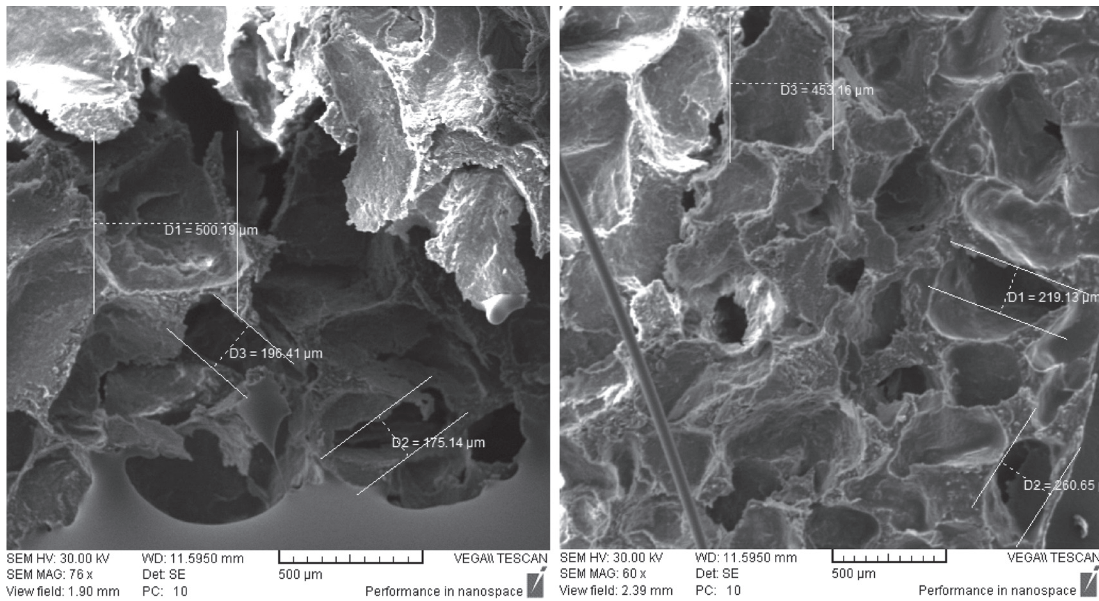


Figure 2. SEM microphotograph of porous hybrid scaffold.

Where W is wet weight (g) and D is dry weight of specimen (g).

Cell culture and MTT assessment

Human osteoblast-like cells (SaOS-2 cell line, Cellular and Molecular Research Center, Tehran University of Medical Sciences) was cultured at 37°C in a humid atmosphere containing 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Germany), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Germany). Trypsin/EDTA (0.05% trypsin/0.53 mM EDTA in 0.1 M PBS without calcium or magnesium) (Invitrogen, Germany) was used for cell passaging. The number of viable cells was evaluated using the MTT assay. For MTT test, scaffolds were sterilized by γ-ray irradiation and normal cells were cultured on the scaffolds at 37°C for 72 h (OC and OC-RGD samples). After incubation, the culture medium was replaced by 150 µl MTT solution (Sigma-Aldrich, USA) and the cells were incubated for 90 min. Thereafter, MTT–formazan was dissolved in DMSO and plates shaken for 15 min. 100 µl of each solution sample was placed in a 96-well plate and optical density (OD) was read at 570 nm using microplate spectrophotometer. The wells without scaffold samples were used as negative control. To correct the possible absorption of MTT or formazan by scaffolds, the same weight of scaffolds used for OC and OC-RGD samples were added to the control samples at the time of adding MTT solution. A blank OD value was reduced from each sample's reading. Cell relative rate (*PGR*) was calculated by Eq.4 (Chen et al. 2008):

$$PGR = \frac{average\ absorbance\ of\ sample}{average\ absorbance\ of\ negative\ control} \times 100 \qquad (4)$$

Assuming exponential cell growth, special growth rate (μ, h^{-1}) was calculated by fitting following formula (Eq.5) to the absorbance data (Tiğli and Gümüşderelioğlu 2008):

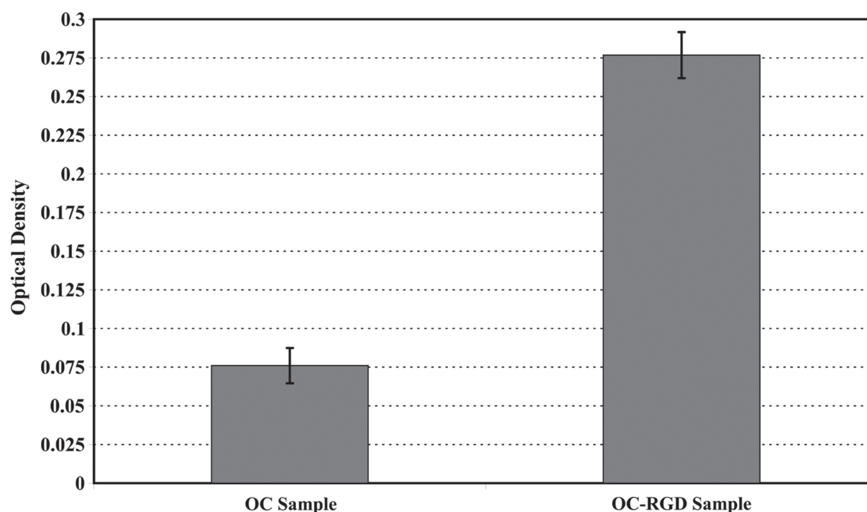


Figure 3. MTT assay of osteoblast cells were cultured on scaffolds. Data were displayed as means ± SD ($n = 5$, $p < 0.001$).

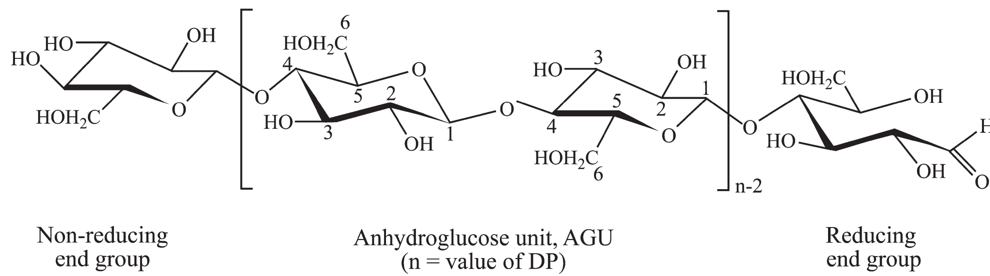


Figure 4. Molecular structure of cellulose (Kamel et al. 2008).

$$\ln \frac{A}{A_0} = \mu (t - t_0) \quad (5)$$

Where A_0 is initial absorbance at t_0 (h), A is absorbance at t (h). Also, growth index was calculated as ratio of cell counts at the end of incubation time to inoculation density.

Statistical method

MTT test and related calculations were done five times. Sample values were expressed as means± standard deviation (SD). A one-way ANOVA on Ranks, Tukey's test posthoc at $\alpha = 0.05$ (using SigmaPlot v11.0 (Systat Software, Inc.)) was carried out to assess significant effect of RGD immobilization on cell proliferation.

Results

Figure 1 shows FTIR spectra of natural and oxidized cellulose powder as well as OC-RGD hybrid. Oxidation of cellulose powder causes some important changes in its FTIR spectrum including change in 400–600 cm^{-1} region, the presence of a strong band at 1700–1750 cm^{-1}, change at 1150–1430 cm^{-1}, and 2800–3000 cm^{-1} besides redistribution of absorption at 3000–3600 cm^{-1}. These spectrum variations are derived by oxidation itself and change in the system of hydrogen bonds (Zimnitsky et al. 2004). Figure 1b clearly shows mentioned peak about 1735 cm^{-1} which is related to carboxyl groups. Oxidized cellulose as same as cellulose is a high biocompatible (Khil et al. 2005) and biodegradable polymer. Enrichment in hydrophilic carboxylic functional groups can lead to improve OC biodegradability (Hersel et al. 2003). Intensity of the band at 1700–1800 cm^{-1} (assigned to C = O stretching of undissociated carboxyl groups) has direct proportion to carboxyl content. Decrease of peak intensity at 1430 and 2800–3000 cm^{-1} (Figure 1b) shows increment of carboxyl content, which is caused by decreasing CH_2OH groups during oxidation and selective oxidation on C-6. Besides, destruction and, therefore, decline in structural order lead to dissipate absorption bands at 1030–1160 cm^{-1} (valence stretching of cyclic C–C and C–O) and 1160–1430 cm^{-1} (bending vibrations of C–OH, C–H and O–H) (Zimnitsky et al. 2004). The carboxyl assigned peak (1735 cm^{-1}) disappeared in spectrum of OC–RGD hybrid (Figure 1c) which was caused by bond formation between surface carboxylic groups of scaffold and amine groups of RGD peptide. RGD reactivation caused transformation of carboxymethyl groups (-COOMe) of protected RGD into carboxyl group (-COOH) which led to appear a peak at ~1735 cm^{-1} again in FTIR spectrum (Figure 1d).

The results of titration test showed DO of 33%–40% and AC of 9%–15% which proved forming of carboxylic acid groups during oxidation. Degree of oxidization represents the number of oxidized hydroxyl groups per anhydroglucose unit of the cellulose sample (Camy et al. 2009). It depends on NO_2 concentration and exposure time of cellulose powder to NO_2 gas. So, increasing of gas concentration and exposure time lead to increase oxidation, thereby DO factor.

Figure 2 shows cross sections of porous hybrid scaffold (OC-RGD sample). Calculated porosity percent was approximately 48%. The higher porosity presented in scaffold, the larger number of cells can come into scaffold and more feasible exchange of nutrient and waste substances. However, increase of porosity can lead to decrease mechanical strength.

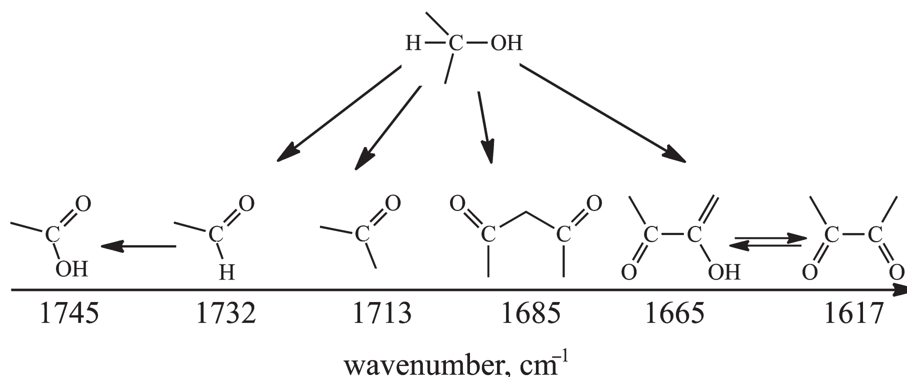


Figure 5. Possible products of cellulose partial oxidation presented as functional groups associated with frequencies of their vibrations (Łojewska et al. 2005).

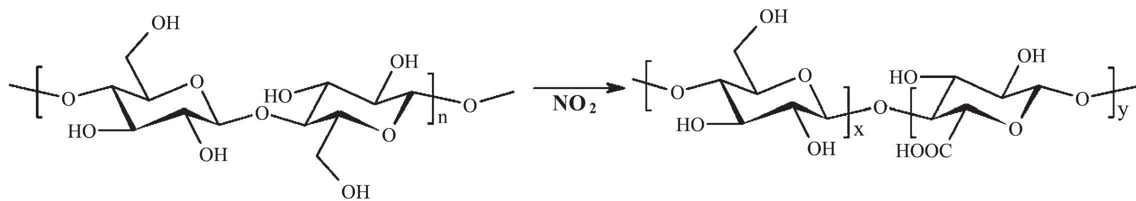


Figure 6. Oxidation process of cellulose by NO_2 gas (Camy et al. 2009).

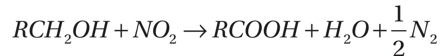
Thus, void volume in load-bearing tissues must be adjusted to allow maintenance of required structural strength besides accommodation of great number of cells (Puppi et al. 2010). So, small amount of PMMA was used to prevent strength reduction, which led to lower porosity percent.

Figure 3 shows OD of OC and OC-RGD samples. Since MTT test measures metabolic activity of cells, the results can be related to number of viable cells called viability. The viable cells, those with functional mitochondrial dehydrogenase, were able to reduce the yellow MTT to a purple formazan product (Idris et al. 2010). From the data in Figure 3, RGD immobilization on OC scaffold had significant influence on proliferation of osteoblast cells. The differences in the mean values among treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p < 0.001$).

Discussion

Cellulose consists of long chain units of anhydro-D-glucopyranose (AGU) with three hydroxyl groups per AGU, with the exception of the terminal ends (Figure 4) (Kamel et al. 2008). Converting some terminal primary alcohol groups of the glucose residues of carboxyl groups may lead to a synthetic polyanhydrocellobiuronide which contains 25% friable and more soluble carboxyl groups. More favorable products have lower carboxyl content (Kamel et al. 2008).

Nitrogen dioxide (NO_2) is the most suitable oxidant to produce OC because secondary hydroxyl groups are protected against oxidation, which results in preventing secondary reaction. Besides, oxidized cellulose preserves acceptable mechanical properties. NO_2 is not available as pure molecular specie and is in equilibrium with its dimer (N_2O_4) (Camy et al. 2009). The proposed stoichiometry of the oxidation reaction is as following:



$$RCH_2OH + NO_2 \rightarrow RCOOH + H_2O + \frac{1}{2}N_2$$

Considering nitric acid formation, the overall reaction can be as following formula:

$$RCH_2OH + 2N_2O_4 \leftrightarrows RCOOH + 2HNO_3 + NO + \frac{1}{2}N_2$$

Most prone C atoms to oxidization occupy 2, 3, and 6 positions in glycopyranose anomer inside the cellulose chain. They give different products on oxidation which are shown schematically in Figure 5. Exposing cellulose powder to nitrogen dioxide gas leads to form COOH groups on C-6 (Camy et al. 2009) (Figure 6).

Esterification, as the transformation of carboxylic acids or their derivatives into esters, causes change in terminal carboxyl groups of RGD into carboxymethyl that cannot react with its amine groups. These amine groups take part in reaction with carboxyl group on the scaffold surface through covalent bonds, thereby grafting RGD peptide on the surface. Eventually, carboxymethyl groups will transform into carboxylic groups after reactivation process. Figure 7 shows reactions required for RGD immobilization.

Surface characteristics like morphology, hydrophilicity, surface energy, and surface charge have significant effects on cell adhesion, cell migration, keeping of cellular phenotype, and intracellular signaling in vitro (Puppi et al. 2010). Macroscopic and microscopic structures of scaffold influence significantly on cellular survival, growth, and propagation. Also, they have great effects on modeling cell shape and genome expressions which lead to protection of natural cell phenotype. The scaffold having interconnected and spread porosities (usually over 90%) besides porous surface and structure can promote ingrowth and reorganization of cell in vitro and supply required space for neovascularizaion in vivo

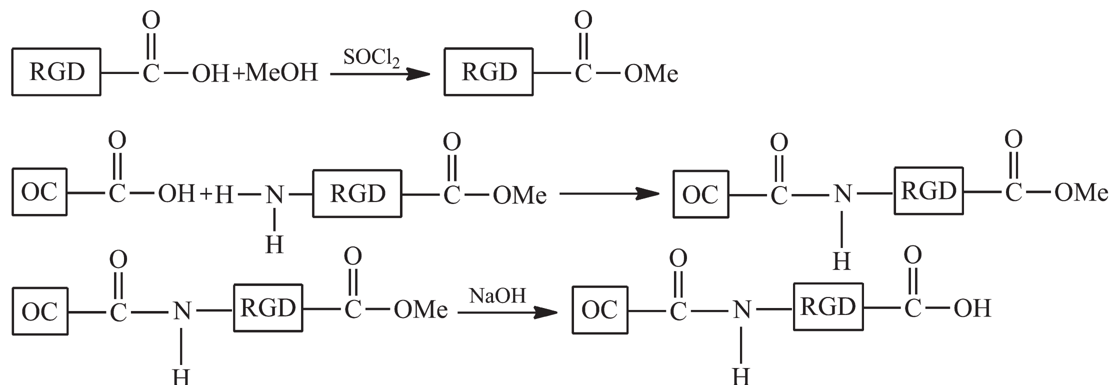


Figure 7. Immobilization reactions for grafting of RGD on scaffold surface.

Table I. Calculated values of PGR, μ and CR using MTT results.

Sample	Cell relative rate (PGR)	Cytotoxicity rank (CR)	Specific growth rate (μ, h^{-1})
OC	71.16	2	0.0282
RGD-OC	101.91	0	0.0461

(Puppi et al. 2010). Also, porosity size must be suitable for osteoblastic cells to enter scaffold. Scaffold containing pores with ideal size is an essential microenvironment for tissue regeneration. Small diameter may provoke pore occlusion by cell, preventing cell penetration within scaffold. Pore size ranging from 75 to 100 μm leads to ingrowth of unmineralized osteoid tissue and smaller pores (down to 10 μm) may be penetrated only by fibrous tissue. Although high internal surface area is necessary to distribute cells truly, increasing pore size leads to decrease internal surface area. So, a compromise must be found. For instance in regenerating bone tissue in vitro, some researches suggested the need for pore size ranging from 200 to 400 μm (Puppi et al. 2010). According to Figure 2, porosities have average size of 300–350 μm, accepted for osteoblasts having 20-30 μm in size. It is expected that the scaffold can perform as a good support for cell accommodation and the following cell proliferation and growth.

Table I presents calculated values of *PGR*, μ, and cytotoxicity rank (*CR*). The cytotoxicity rank is 0, 1, 2, 3, 4, and 5 when *PGR* is ≥ 100, 75–99, 50–74, 25–49, 1–24, and 0, respectively (Chen et al. 2008). The values also prove enhancement of biocompatibility of OC scaffolds by immobilizing RGD.

Beside of no cytotoxicity for oxidized cellulose (Khil et al. 2005) and obtaining $CR = 2$ by using MTT results, it is obvious that RGD immobilization caused decrease in *CR* value (consequently, biological improvement) and 63.48% increase in specific growth rate.

Conclusions

FTIR examination proved oxidation of cellulose powder and formation of carboxyl groups on scaffold surface. Also, bond formation between amine groups of RGD and surface carboxylic acid groups of scaffolds was evidenced by FTIR. According to titration test, degree of oxidation and carboxylic acid content of OC scaffold were 33%–40% and 9%–15%, respectively, which confirmed formation of carboxyl group again. SEM study showed 3D porous structure having well-created porosities in size for scaffolds. Average porosity size and percent were 300–350 μm and 48%, respectively. Viability or number of viable cells, cytotoxicity rank, and specific growth rate of OC-RGD scaffold increased significantly compared with OC scaffold which confirmed positive effect of RGD immobilization on biocompatibility of scaffold.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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