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Erstveröffentlichung in / First published in:

Textile Research Journal. 2014, 84(10), S. 1084 – 1094 [Zugriff am: 07.08.2019]. SAGE journals. ISSN 1746-7748.

DOI: <https://doi.org/10.1177/0040517513515315>

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Net Shape Nonwoven: a novel technique for porous three-dimensional nonwoven hybrid scaffolds

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Textile Research Journal
2014, Vol. 84(10) 1084–1094
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sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/0040517513515315
trj.sagepub.com

Abstract

Textile structures made of biocompatible, osteoconductive and resorbable chitosan-filaments provide excellent preconditions as scaffolds for Bone Tissue Engineering applications. The novel Net Shape Nonwoven (NSN) technique that enables short fibers to be processed into three-dimensional net-shaped nonwoven structures with adjustable pore size distributions is described. NSN scaffolds made of pure chitosan fibers were fabricated. NSN hybrid scaffolds for improved initial cell adhesion were realized by combining the NSN technique with electrospinning and dip-coating with collagen, respectively. Scanning electron microscopy and liquid displacement porosimetry revealed an interconnecting open porous scaffold structure. The novel chitosan-hybrid scaffolds provide proper conditions for adhesion, proliferation and differentiation of the seeded human bone marrow stromal cells, proving that they are suitable for usage in hard-tissue regeneration.

Keywords

fiber based scaffold, biomaterials, chitosan, tissue engineering

Scaffolds for Bone Tissue Engineering (BTE) have been widely investigated in the past decades. The optimal choice of material and scaffold architecture still remains an unsolved issue despite intensive research activities in this area.¹ The aim to resemble the extracellular matrix (ECM) of bone tissue in a three-dimensional (3D) porous environment is a challenge that has led to a diversity of scaffold manufacturing approaches.² For the creation of 3D interconnected porous scaffolds that provide proper conditions for cell ingrowth, techniques based on solid free forming (SFF), for example selective laser sintering, 3D printing or fused deposition modeling (FDM), have been widely studied,³ allowing the creation of graded porous structures.⁴ In these techniques ceramic materials, which are rigid and inelastic after sintering, are commonly used.² Another approach that has been extensively studied for the creation of an ideal scaffold are nanofibrous meshes, mostly produced using the electrospinning technique.^{5,6} Nanofibrous meshes can be designed to very closely mimic the ECM and therefore provide favorable conditions for cell-attachment, proliferation and differentiation.^{7,8} Although much attention has been put on improving

the physical and chemical surface properties of electrospun scaffolds in order to enhance their cellular compatibility, a major concern of electrospun scaffolds is that they are limited to thin two-dimensional (2D) constructs and thus do not provide the necessary 3D environment and mechanical properties.^{7,9} In order to combine the advantages of 3D scaffolds made of synthetic polymers with the improved cellular compatibility of nanofibers, a common approach is the fabrication of hybrid scaffolds, in which both nano- and microdimensional elements are present.¹⁰ This can be achieved through combining melt- and solution electrospinning¹¹ or through the combination of

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additive manufacturing (AM) techniques such as FDM together with solution electrospinning.¹² Due to excellent surface-to-volume ratio and their porous fibrous structure, textile fabrics can be designed to meet the prerequisites for BTE scaffolds.¹³ Although the processing of fibers into spatial constructs is the key competence of textile technology, only very few scaffolds fabricated using textile processing methods are reported.^{14–17} The natural cationic polysaccharide chitosan (CS) has been widely investigated for BTE applications because of its biocompatibility, biodegradability, its ability to support wound healing and osteoconduction and its antimicrobial properties.¹⁶ To be able to exploit those favorable properties in a single-component, structurally stable system, it was necessary to develop microfibers that can be subsequently processed in textile manufacturing techniques.¹⁸ Collagen type 1 has been widely used to improve biological activities on scaffolds for BTE, because it is the major constructional element of the ECM of bone, promotes cell adhesion and is biodegradable.¹⁹ In this paper the novel manufacturing method Net Shape Nonwoven (NSN) for 3D nonwoven structures is introduced. This SFF technique makes it possible to process textile fibers into three-dimensionally shaped nonwovens. Hybrid structures are realized by collagen functionalization and by incorporating electrospun nanofibers throughout the scaffold.

Methods

Materials

CS with a deacetylation degree of 90% and viscosity of 50 mPas (1 %-wt. CS in 1 %-wt. acetic acid) was provided by Heppe Medical Chitosan GmbH, Germany. Porcine gelatin was bought from Sigma Aldrich, USA. Bovine type 1 collagen was provided by GfN GmbH, Germany. 0.1M tris(hydroxymethyl)aminomethane (TRIS) buffer solution was produced from TRIS powder (Carl Roth, Germany). The crosslinker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was provided by Sigma Aldrich, USA. Glacial acetic acid (AcOH) was purchased from Merck, Germany. CS microfibers with average fiber diameters of approximately 20 μm were produced using an in-house semi-industrial wet spinning machine (Fourné Polymertechnik GmbH, Germany) from CS dopes dissolved in acetic acid solutions.

NSN technique

The NSN technique is a SFF manufacturing technique that processes textile fibers into 3D nonwovens without the usage of negative molds or forging dies, thus enabling the fabrication of textile structures with the versatile processing options of SFF techniques. Figure 1 describes the basic principle of the NSN.

Figure 1. Basic scheme of Net Shape Nonwoven production technique.

As basic material for the sample fabrication, a homogeneously distributed web of short fibers is set onto the fabrication slide by a fiber deposition mechanism (Figure 1(a)). Depending on the fiber material (e.g. thermoplasts, polysaccharides, metals), a bonding device that is mounted onto an X-Y stage creates a permanent linkage between the fibers in a defined area (Figure 1(b)). Table 1 exemplarily shows possible bonding mechanisms for different fiber types. After each fabricated layer, the fabrication slide is moved down by the thickness of one layer. The steps “fiber deposition” and “fiber bonding” are being repeated until the complete 3D nonwoven is fabricated. At the end of the production cycle, the fabrication slide is moved up and the scaffold, which sits within unbonded fibers, can be removed (Figure 1(c)).

For the fabrication of hybrid nonwovens made of pure CS microfibers and nanofibers, which may be used as scaffolds for BTE, the NSN machine was set up as displayed in Figure 2. In a preliminary step, CS microfibrinous filament yarns were cut into short fibers with the length of 0.5–2 mm. The short fibers are deposited as described above (Figure 2(a)). As cohesive bonding media, 0.5 %-vol. acetic acid is applied by means of a micro dispensing unit “MD-K” (microdrop technologies GmbH, Germany), which creates droplets of approximately 100 µm diameter in an adjustable frequency according to the requirements of the fabricated scaffolds (Figure 2(b)). Once wetted, the surface of an individual CS fiber gets partially dissolved. Fibers of the same or a subsequent fiber layer contact the partially dissolved spots, thus creating a cohesive bonding

Table 1. Possible bonding mechanisms for Net Shape Nonwoven fabrication

Bonding mechanism/bonding media	Fiber type
Reactive synthetic adhesives, e.g. ultraviolet-curing acrylates	Polyester, polyamide
Bioadhesives, e.g. gelatin	Poly lactide
Partial dissolving of fiber surface, e.g. dilute acetic acid	Chitosan
Thermal activation, e.g. laser activation	Titanium

Figure 2. Description of the Net Shape Nonwoven technique for chitosan hybrid scaffold production, showing schematic drawings (bottom) and images of their respective machine parts (top). Fiber deposition (a), application of 0.5 %-vol. AcOH as bonding media (b), electrospinning (c).

Table 2. Fabricated Net Shape Nonwoven scaffolds

Sample	Fiber length [mm]	Nanofiber functionalization	Collagen functionalization
NSN_1mm	1	–	–
NSN_2mm	2	–	–
NSN_nano_1mm	1	Yes	–
NSN_coll_1mm	1	–	Yes

Figure 3. NSN_1mm scaffold fabricated in exemplary three-dimensional shape.

between the individual CS fibers. The hybrid micro-nanoscaled architecture of the NSN scaffolds is obtained by an electrospinning process, which creates a thin nanofibrous mesh onto the microfiber layer (Figure 2(c)). This nanofiber functionalization may be incorporated continuously (after each layer of microfibers) or selectively. In this work, gelatin nanofibers were applied using electrospinning parameters as described in the *Nanofiber functionalization* section. The result of this production technique is a 3D hybrid nonwoven fabric comprising randomly orientated CS microfibers and gelatin nanofibers that are cohesively bound together.

Nanofiber functionalization

To generate hybrid scaffolds with micro- and nanoscaled components, the microfibrinous NSN scaffolds were functionalized with nanofibers. This was obtained through gelatin electrospinning. Therefore, gelatin solutions of 15%-wt. gelatin dissolved in 60%-vol. AcOH were prepared. The spinning solution was fed through a G21/0.8mm blunt needle. Electrospinning was conducted using a voltage of 24 kV and a working distance of 15 cm.

Figure 4. Chitosan microfibrinous Net Shape Nonwoven scaffolds fabricated from different fiber lengths: NSN_1mm (a); NSN_2mm (b).

Collagen functionalization

An alternative way to creating hybrid scaffolds is the collagen functionalization of microfibrinous NSN scaffolds. In order to investigate the influence of collagen coating on the scaffold properties, microfibrinous NSN scaffolds were coated with bovine type 1 collagen by dipping the scaffolds into a solution of 2 mg/ml collagen dissolved in 0.1M TRIS buffer solution with pH 7.4, which was placed in an ultra-sonic bath for approximately 10 s. The dip-coated scaffolds were freeze-dried and subsequently chemically cross-linked using the water-soluble carbodiimide EDC as described elsewhere.²⁰

Characterization

Morphology. The morphology of the NSN scaffolds was investigated using a Scanning Electron Microscope (SEM) XL 30 ESEM (Philips Electron Optics, NL).

Long-time stability. The long-time stability of the NSN scaffolds in different media (demineralized water, phosphate-buffered saline (PBS) and cell-culture

Figure 5. Bonding points of NSN_1mm scaffolds, demonstrating the cohesive inter-fiber bonding. Different magnifications: 350 $\times$ (a); 650 $\times$ (b); 800 $\times$ (c).

media (Dulbecco's modified eagle medium, dMEM), respectively) was tested by immersing the scaffolds into the media and storing them at 37°C for 28 days. The fiber swelling rate S was determined by Equation (1):

$$S = \left(\frac{T_i}{T_d} - 1 \right) \cdot 100 \% \quad (1)$$

Figure 6. NSN_nano_1mm scaffolds. The gelatin nanofibers create a thin web within the chitosan microfibers.

where S is the fiber swelling rate [%], T_i is the fiber thickness after 24 h immersion in testing liquid [μm] and T_d is the thickness of dry fibers after conditioning in 20°C, 65% relative humidity for 48 hours [μm].

Compressive deformation behavior. Compressive deformation behavior under both static and cyclic load was investigated using a standard material testing machine (Zwick Z2.5, Zwick GmbH, Germany). Specimens in the size of 10 mm $\times$ 10 mm $\times$ 10 mm (length $\times$ width $\times$ height) were produced using the NSN technique. Dry conditioned scaffolds (20°C, 65% relative humidity) as well as scaffolds immersed in cell-culture media (dMEM) were tested. The displacement speed was 0.65 mm/s for static testing and 1.5 mm/s for cyclic testing. An initial load of 0.3 cN was applied in order to flatten single fibers protruding from the scaffolds. Static testing was performed until a deformation rate of 75%. Cyclic testing was performed by repeating 25 cycles between deformation rates of 25% and 60%, respectively.

Porosimetry. The pore size distribution of the unfunctionalized and collagen functionalized CS NSN scaffolds

Figure 7. Collagen functionalized NSN_coll_1mm scaffold at different magnifications, showing the fibrous collagen coating on the chitosan fibers: 80× (a); 200× (b); 500× (c).

was measured using the liquid displacement technique (Pore Size Meter PSM 165, TOPAS GmbH, Germany) following ASTM E1294-89 and ASTM F316-03, which are suitable testing methods for voluminous fabrics such as nonwovens and enable compression-less mounting of the samples. As a capillary constant, 28.6 was used. As a testing fluid, a perfluorocarbon (surface tension $\rho_1 = 16 \text{ mN/m}$), which secures complete wetting of the scaffolds without swelling, was used. The sample

Figure 8. Chitosan Net Shape Nonwoven (NSN) scaffolds after immersion in phosphate-buffered saline (a) and Dulbecco's modified eagle medium (b) and storage at 37°C for 28 days; dry NSN chitosan scaffold (c).

Table 3. Porosity values of Net Shape Nonwoven scaffolds

Sample	Overall porosity [%]	Median pore size [μm]	Pore size range [μm]
NSN_1mm	96.4	118 $\pm$ 15	35–300
NSN_2mm	98.5	137 $\pm$ 13	40–400
NSN_coll_1mm	91.8	25 $\pm$ 5	15–55

size for porosity measurements was $12 \text{ mm} \times 12 \text{ mm} \times 2.5 \text{ mm}$ (length $\times$ width $\times$ height). The overall porosity P_{res} (Equation (2)) of the scaffolds was calculated from the scaffold volume, the resulting density of the scaffolds and the density of the bulk raw materials (CS-microfibers: 1.7 g/cm^3 and collagen 1.3 g/cm^3):

$$P_{res} = \left(1 - \frac{m_{scaff}}{V_{scaff} \cdot (\rho_{bulk})} \right) \cdot 100 \% \quad (2)$$

where P_{res} is the overall scaffold porosity [%], $\frac{m_{scaff}}{V_{scaff}}$ is the resulting scaffold density [mg/mm^3] and ρ_{bulk} is the resulting bulk density of material combination [mg/mm^3].

Cell-culture experiments

To gain fundamental knowledge about the cellular behavior on the novel NSN scaffolds, cell-culture experiments were performed using selected samples. Therefore, microfibrinous scaffolds and collagen-coated microfibrinous scaffolds, both made of 1 mm fiber length, were used. The unfunctionalized CS scaffolds (NSN_1mm) and collagen-functionalized CS scaffolds (NSN_coll_1mm) were seeded with a cell number of $3 \cdot 10^4$ human bone marrow stromal cells (hBMSC). The adhesion and proliferation of the seeded cells were determined by measuring the total activity of

Figure 9. Pore size distribution of Net Shape Nonwoven chitosan scaffolds; unfunctionalized scaffolds versus collagen functionalized scaffolds; 1 mm fiber length.

lactatdehydrogenase (LDH) using the LDH Cytotoxicity Detection Kit (Takara, Otsu, Shiga, Japan). The LDH activity was correlated with the cell number using calibration lines of cell lysates with known cell numbers. The differentiation of the hBMSC to the bone-building cell osteoblasts was confirmed by the measurement of alkaline phosphatase (ALP). The distribution of the cells seeded and cultivated on the NSN scaffolds were visualized using a SEM and confocal laser scanning microscopy (cLSM). Therefore, a fluorescence staining of the cytoskeletal actin as well as the nuclei of the fixed cells was performed using AlexaFluor 488-Phalloidin (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI, Sigma).

Results and discussion

The sample designation of the fabricated samples is given in Table 2.

Scaffold morphology

Figure 3 shows a scaffold fabricated from pure CS microfilament using the NSN technique without further processing. The combination of a cylinder on top of a cuboid demonstrates the ability for fabricating 3D-free-formed samples.

SEM images of the NSN scaffolds fabricated from different fiber lengths reveal their open porous structure with interconnected pores (Figure 4). Figure 5 shows

bonding points between individual fibers at different magnifications. The cohesive inter-fiber bonding becomes clearly visible.

The nanofiber functionalization for the fabrication of NSN hybrid scaffolds is shown in Figure 6. The application of nanofibers between each layer of CS microfibers leads to a fine nanofibrous web that can be beneficial for cell adhesion. This nanofiber functionalization may also be conducted using CS electrospinning solutions. Collagen functionalization leads to a complete surface covering of the CS microfibers (Figure 7). The interconnectivity of the pores is maintained, yet the pore size is reduced. The collagen coating exhibits a very fine fibrillar structure similar to ECM (Figures 7(b) and (c)).

Long-time scaffold stability and fiber swelling

The CS microfibrous 3D scaffolds exhibit excellent long-time stability after immersion and storage at 37°C for 28 days (Figure 8). No shrinkage or deformation could be observed. Once soaked with a liquid, swelling of the CS fibers was observed. Swelling rates of 42% (dMEM), 54% (PBS) and 70% (H₂O), respectively, were determined. The fiber swelling of the CS microfibers depends on the interface interactions between fibers and their surrounding liquid. The NSN scaffolds bear further processing (e.g. ultra-sonic treatment), maintaining their original shape and form stability.

Figure 10. Deformation behavior of NSN_1mm scaffolds. Dry samples (a) show areas of elastic deformation, plastic deformation and compression, respectively. Samples immersed in Dulbecco's modified eagle medium (b) show areas of elastic deformation and plastic deformation / compression, respectively.

Porosimetry

Table 3 shows the results of the pore size measurements and the calculation of the overall porosity. With overall porosities of well above 95% and median pore sizes in the range of 118–137 μm , the open, interconnective

porous structure of the NSN CS scaffolds is confirmed. The collagen functionalization leads to largely reduced pore sizes, yet the overall porosity stays above 90%. This indicates a very fine collagen layer that forms a narrow fibrillar network. The unfunctionalized scaffolds show a broad pore size distribution, whereas the

Figure 11. Scanning Electron Microscope image of a human bone marrow stromal cell on the chitosan Net Shape Nonwoven scaffold 24 h after seeding.

pore size distribution turns narrow after collagen coating. The presented pore size values refer to non-swollen scaffolds. When the scaffolds are immersed in cell-culture media, the pore size is reduced relative to the fiber swelling rate. Two typical histograms of pore size distributions are displayed in Figure 9.

Compressive deformation behavior

As a basic characterization for the novel NSN structures, fundamental deformation curves under static load were recorded for dry scaffolds and scaffolds immersed in dMEM (Figure 10). These deformation curves depend on the material and structural specific failure behavior of the fibrous scaffolds. The imparted compressive load results in two different effects: (a) bending of the individual fibers and (b) breaking of bonding points between the fibers. Fiber bending accounts for the elastic deformation of the scaffolds, whereas breaking of bonding points leads to plastic deformation. Immersion in dMEM or other buffer salines leads to fiber swelling (see “long-time scaffold stability”).

Dry scaffolds show a linear increase of compressive load until approximately 10% deformation, which may be affiliated to reversible bending of the fibers. Fiber bending is followed by subsequent breaking of the bonding points and compression of the scaffold (Figure 10(a)). The swollen fibers possess less stiffness, thus dramatically reducing their resistance against bending. As an effect, the immersed NSN scaffolds show low resistance against deformation, yet provide high elasticity. A linear increase of compressive load until approximately 40% is registered (Figure 10(b)). The elasticity of the immersed NSN scaffolds is a prerequisite for usage as “press-fit” implants in surgery.

Figure 12. Laser scanning microscopy images of human bone marrow stromal cells on chitosan Net Shape Nonwoven scaffolds functionalized with collagen (a) 7 days and (b) 14 days after seeding. The actin skeleton is visualized green, the nucleus blue. The chitosan fibers also appear blue. (Color online only).

Since scaffolds for regenerative medicine are always applied in wet conditions, all subsequent material testing is to be done in wet conditions. Cyclic testing gives information about the elastic and plastic deformation and proves the high elasticity of the NSN scaffolds.

Cell-culture experiments

The seeding efficiency with hBMSC was about 15% for both the NSN_1mm scaffolds and the collagen functionalized NSN_coll_1mm scaffolds. The cells adhered on the NSN scaffolds 24 hours after seeding (Figure 11).

Figure 13. Proliferation rate of the human bone marrow stromal cells on the chitosan Net Shape Nonwoven (NSN) scaffolds (NSN_1mm), the collagen-coated chitosan NSN scaffolds (NSN_coll_1mm) and on polystyrene. The cell number was determined by the measurement of lactatdehydrogenase. The cells were cultured in Alpha-MEM (-) and Alpha-MEM with osteogenic supplements (+).

The adhesion and proliferation of the hBMSC on NSN_1mm_coll is visualized in Figure 12. After 28 days of cultivation on the NSN scaffolds without osteogenic supplements, the cells showed a proliferation rate of 4.1 ± 0.7 on NSN_1mm and 6.9 ± 0.7 on NSN_coll_1mm. The cell number and proliferation rate was determined by LDH measurement (Figure 13). The cultivation of cells on polystyrene (PS) is used as a standard method to evaluate the physiological cell behavior. Here the cells seeded on PS are used to measure the influence of the induced osteogene differentiation towards cell proliferation to evaluate this effect to the cells cultivated on the different treated CS scaffolds. When osteogenic supplements were added, no differences in the proliferation rate on the two scaffold modifications could be observed. One potential reason for this could be the proliferation-enhancing effect of the osteogenic supplement. The differences in the proliferation rate between the cells cultivated on NSN_1mm or on NSN_coll_1mm scaffolds demonstrate that the collagen coating leads to improved conditions for cell proliferation. The cell differentiation towards the osteoblast lineage was confirmed by the relative ALP activity of the cells (data not shown). The increased proliferation and differentiation of the hBMSC in the presence of collagen can be explained by the higher specific surface area and the similarity to ECM and bone.

Conclusion and outlook

The presented novel NSN technique sustains the fabrication of porous 3D hybrid scaffolds that can be used in hard-tissue regeneration. Variation of fabrication parameters may allow the realization of scaffolds with adjusted pore sizes. The elasticity of the immersed scaffolds is advantageous for their end-use. The NSN CS scaffolds provide proper spatial conditions for the adhesion, proliferation and differentiation of hBMSC and exhibit good long-time stability in various buffer salines. A nanofiber functionalization throughout the scaffold was realized. First cell-culture experiments of the collagen functionalized hybrid scaffolds showed promising results. Cell-culture experiments with gelatin and CS nanofiber functionalized NSN scaffolds will be performed and published in a separate study in order to prove the favorable conditions of nanofibers in a 3D porous network.

As an additional remark, the fabrication of scaffolds for BTE is only one exemplarily application for the NSN technique. Depending on the fiber material and the bonding media, nonwovens with completely different properties could be realized. Hence, the NSN technique may be considered a novel platform technique that may be used for a wide range of applications.

Funding

This research project (DFG CH 174-24/1) is supported by “Deutsche Forschungsgemeinschaft”.

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