# 3D Nanostructural Analysis of Biomaterials by Scanning Probe Nanotomography

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## ABSTRACT

Scanning probe nanotomography is based on serial section ultramicrotomy and consequent AFM measurements carried *in situ* by one integrated system. It was used to study the 3D structure and characteristics of nanoporosity and pore interconnectivity of biodegradable scaffolds made of recombinant spidroin 1 (rS1/9) and fibroin dedicated for regenerative medicine applications. Significant nanoporosity (24%) and pore percolation was detected for rS1/9 scaffolds. Fibroin scaffolds show negligible nanoporosity (0.5%) and pore interconnection. It is considered crucial for higher *in vivo* efficiency of tissue regeneration observed on rS1/9 scaffolds.

***Keywords***: nanotomography, percolation, biocompatible scaffolds, fibroin, spidroin.

## 1 INTRODUCTION

Modern research and development of new biological and bioinspired materials for tissue engineering and regenerative medicine applications request for nanometer scale characterization of local structure and morphology with high-resolution microscopy techniques. Information on the local nanoscale 3D volume organization of complex biomaterial systems becomes more and more important; in fact better understanding of essential parameters determining interface organisation in the bulk of biomaterial systems, parameters of multiscale porous networks and distribution of nanocompounds in the volume, and their influence on the macroscopic physical and biological materials properties makes access to nanoscale volume information imperative. However standard nano-microscopy approaches (SEM and SPM) probe mainly the sample surface. SPM can be considered as a non-destructive surface characterization technique for biomaterial analysis [1, 2], which, contrary to the electron microscopy, does not induce any radiation damage of the sample surface. In order to empower SPM with capabilities of 3D volume analysis we have been developing a new methodology and unique instrumentation for 3D reconstruction of the nanoscale volume organization of soft matter – scanning probe nanotomography (SPNT) [3]. It combines SPM with an ultramicrotome [3] or cryoultramicrotome [4] and can be applied for serial section tomography of a wide range of soft biological and polymer materials in the native state. Three-dimensional structures of the objects in the volume are reconstructed via integration of stack of consecutive surface images after ultrathin sections. The integrated instrument enables direct SPM measurements of a block face surface immediately after each sectioning by the ultramicrotome knife, therefore resolution in depth direction is limited only by the minimal thickness of sections removed from the block face surface and can go down to 10 nm. It is also worth notice that this technique has no principal limit on the thickness of the volume analyzed and analysis of several micrometer thick volumes is possible. Integration of SPM with cryoultramicrotome permits in situ measurements of the sample surface after cryo sectioning without any modification of the sample ultrastructure or transfer of the sample. It provides unique capabilities to study native structures of soft and hydrated materials and biological systems stabilized by cryofixation. The SPNT technology was successfully applied for study of 3D structure of biological materials and different advanced materials like liquid crystal/quantum dot [5], polymer/nanotube [6] and polymer/graphene nanocomposites [7].

Here we present new results of SPNT 3D ultrastructure studies of such biomaterial systems as biodegradable polymer cell scaffolds based on the recombinant protein rS1/9 (an analog of spidroin 1, protein of dragline silk produced by *Nephila clavipes* spiders) expressed in yeast and fibroin from natural silkworm (*Bombyx mori*) silk [8].

## 2 MATERIALS AND METHODS

### 2.1 Preparation of scaffolds

Natural silk from *B. mori* cocoons was boiled for 1 h in 0.03 M $NaHCO_3$, then it was thoroughly rinsed with distilled water to remove the glue-like sericin proteins and wax. The polymer protein rS1/9 was purified as described previously [8]. Porous scaffolds were fabricated from the natural silkworm fibroin and rS1/9 by the salt leaching technique. The polymer proteins were dissolved in a solution of 10% lithium chloride in 90% formic acid. Proper amounts of dry NaCl particles of 200–400 um in diameter were added to the polymer solutions with concentration up to 110 mg per 50 mcl. The shaped and dried scaffolds were treated with 96% ethanol. NaCl particles were removed by solving in distilled water. The samples of scaffolds were embedded in epoxy resin for ultramicrotome sectioning and AFM measurements.

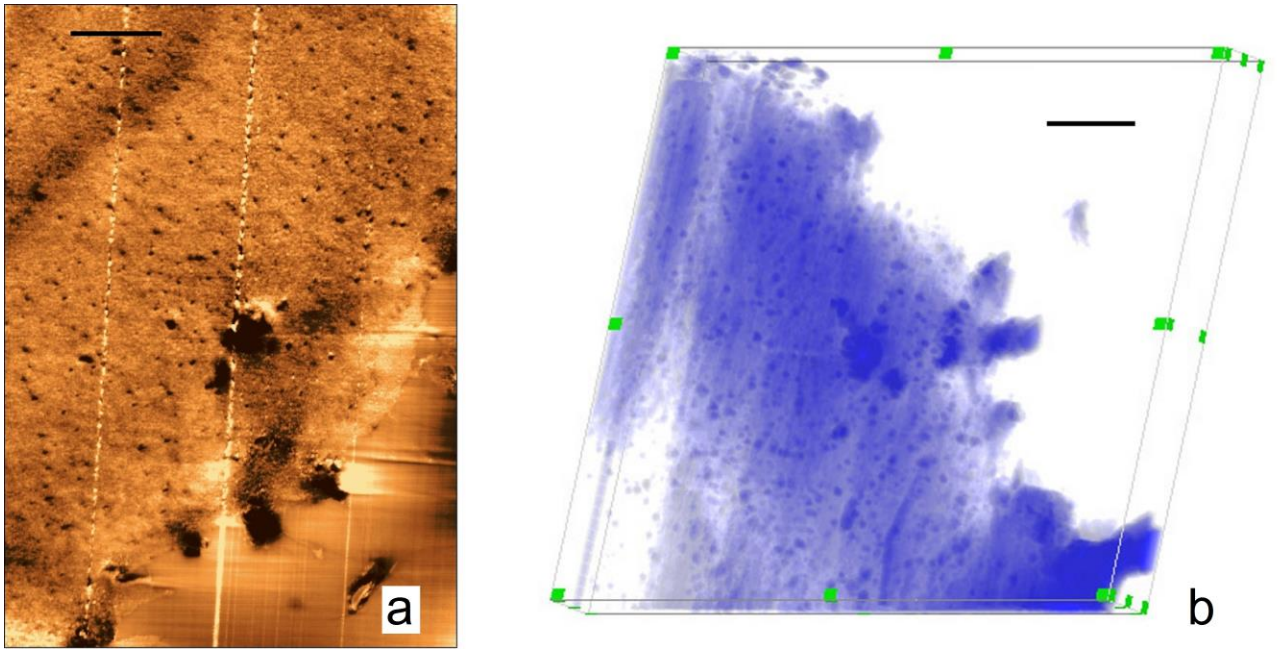


Figure 1: a) AFM topography image of fibroin-based scaffold surface after ultramicrotome section, 21.0×30.0 um$^2$, height variation 35 nm; b) 3D reconstruction of macropore wall structure in fibroin-based scaffold (20 sections, 45.0×32.8×1.8 um$^3$). Scale bars 4 um.

## 2.2 Scanning probe nanotomography

For scanning probe nanotomography studies we have used NTEGRA-Tomo system (NT-MDT Co., Russia). This system comprises an SPM combined with a Leica EM UC6NT ultramicrotome (Leica Microsystems GmbH, Austria). The system is allowing to scan the sample surface with the SPM tip immediately after sectioning with help of SPM measuring head directly mounted on the ultramicrotome knife holder [3]. Ultra AFM 35 diamond knife (Diatome AG, Switzerland) was used for sectioning of the samples. AFM measurements were carried out in the semicontact mode with use of cantilever tips NSC14 (Micromasch, Estonia) with a characteristic resonant frequency of about 160 kHz and tip radus smaller than 10 nm. Series of consequent AFM images of scaffold macropore wall cross-sections with were acquired with 70 nm sectioning between images. 3D structures were visualized and analyzed with use of ImagePro AMS 6.0 software package with 3DConstructor option (MediaCybernetics, Inc., USA)

# 3 RESULTS AND DISCUSSION

One of the main concerns of present study is quantitative investigation of size, density and interconnectivity of nanopores in the volume of scaffolds. The main applicatoion of such scaffolds is use as implanted biodegradable cell matrices for regeneration of the native tissue. Parameters of porosity on micro- and nanoscale in scaffolds are critical for the efficacy of tissue integration; an interconnected structure is a prerequisite for homogeneous cell distribution and effective tissue intergrowth *in vivo*, as this type of structure facilitates active gas exchange, nutrient supply, and proper metabolic waste removal [9].

Both fibroin and rS1/9 scaffolds produced by the salt leaching technique have macropores which are approximately 200–400 um diameter. Macropores are divided by macropore walls with thickness in the range of several tens of microns. For investigation of 3D structure of scaffolds macropore walls by SPNT technique we have performed serial ultramicrotome sectioning and consequent *in situ* AFM measurements for both scaffold samples. We found that nanoscale porosity in the bulk of macropore walls is dramatically different for these scaffolds.

Fig. 1a shows typical AFM image and SPNT 3D reconstruction of fibroin-based scaffold macropore wall. We can detect individual nanopores with dimensions in range from 30 to 200 nm. Integral nanoporosity of fibroin macropore walls do not exceed 0.5%. 3D SPNT reconstruction obtained from 20 consequent 45.0×45.0 um$^2$ AFM images (Fig. 1b) shows that most of the nanopores are not connected with each other.

In the bulk of rS1/9 scaffold macropore walls we do observe the nanopores of similar dimensions but the integral porosity degree is much higher (~24%) than in fibrion ones. Moreover most of nanopores seem to be connected with others (Fig. 2). But only 3D reconstruction analysis can provide correct estimation of interconnectivity and percolation in the nanopore system. 3DContructor software enabled us to vizualize clusters of interconnected nanopores in 3D volume by building of isosurfaces of pore borders and to calculate parameters of pore clusters. Fig. 3 shows visualization of nanopore percolation clusters in rS1/9 scaffold macropore wall and visualization of nanopores in the similar volume of fibroin-based scaffold. We have calculated that 35.3% (volume fraction) of all nanopores are interconnected in such clusters. Dimensions of these percolation clusters range from 1.5 to 6.0 um.

This difference in nanoporosity in fibroin and rS1/9 scaffolds may be caused by differences in molecular structure of these proteins as they have different molecular masses (94.3 kDa for rS1/9 and 370 kDa for fibroin) and different amino-acid sequences (the (GAGAGS)n motif found in the fibroin and poly-A blocks and alternation of hydro-phobic and hydrophilic sections found in rS1/9 protein). These differences can cause different nanopore formation in scaffolds at the time of contact with the ethanol.

According to the results of 3D analysis we can expect that nanoscale permeability and percolation in macropore walls of rS1/9-based scaffolds may positively effect to *in vivo* cell proliferation and corresponding tissue regeneration. That corresponds with the *in vivo* rat bone tissue regeneration studies that were carried out earlier [8].

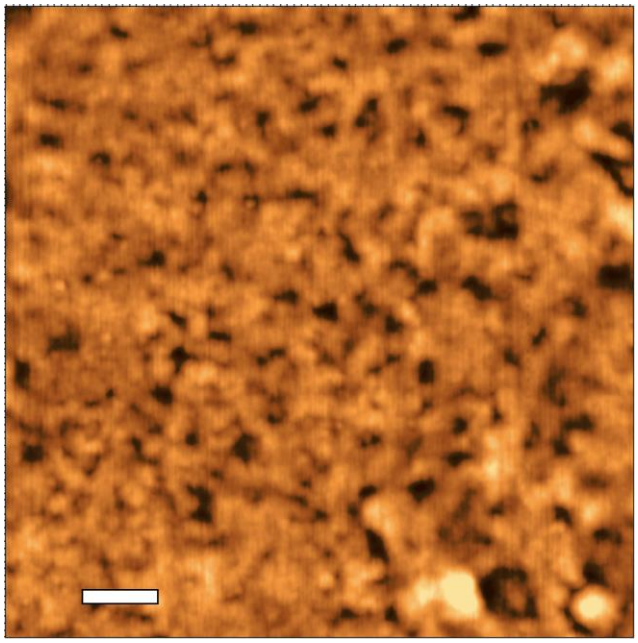

Figure 2: AFM topography image reconstruction of rS1/9-based scaffold macropore wall after ultramicrotome section, 1.8×1.8 $um^2$, height variation 25 nm, scale bar 200 nm.

## 4 CONCLUSION

We found that nanoscale porosity is much more developed in rS1/9 scaffolds than in fibroin ones (24% vs. 0.5%). High degree of pore interconnectivity and percolation what can positively effect to *in vivo* cell proliferation and corresponding tissue regeneration on rS1/9 scaffolds. That is one of the indicatives, together with biocompatibility, slow biodegradability and mechanical strength, that recombinant spidroin 1 has bright prospectives as a biomaterial for a number applications in regenerative medicine.

Presented nanotomography technique applied to analysis of micro- and nanoporous materials can provide resolution of tens of nanometers in all three dimensions. Further development of SPNT technique may provide new insights about 3D organization of biomedical materials (tissue scaffolds and drug delivery microsystems), biological objects (tissues, cells, and microorganisms) and other nanocomposite materials.

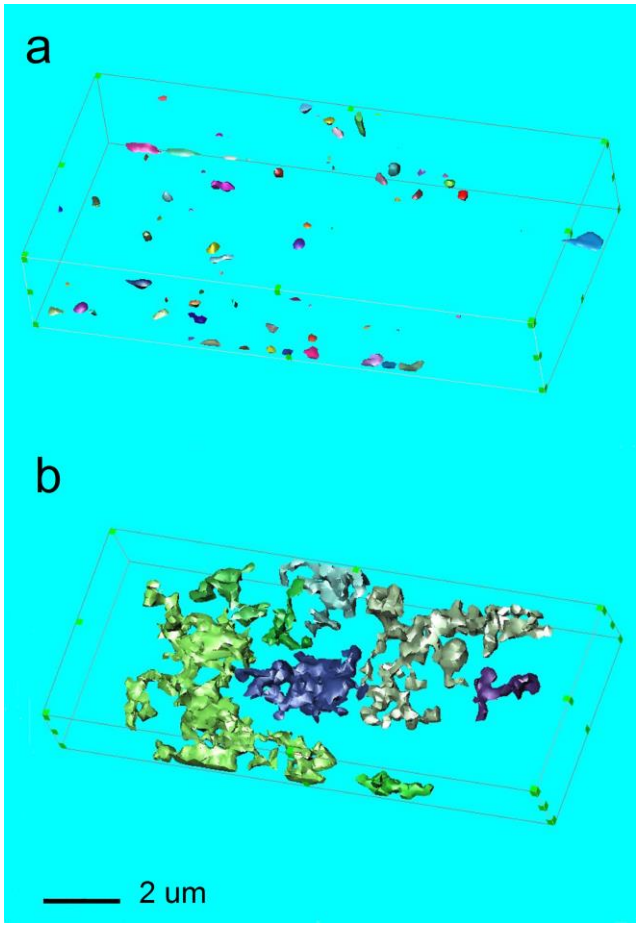


Figure 3: a) 3D reconstruction of nanopores in mcropore wall of the fibroin-based scaffold (6.36×3.18×1.0 $um^3$); b) 3D reconstruction of interconnected clusters of nanopores in mcropore wall of the scaffold rS1/9-based scaffold (7.54×3.28×1.0 $um^3$).

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