

# Preparation of Bacterial Nanocellulose-Hydroxyapatite Composite

Anna Kutová\*, Ondřej Kvítek, Klaudia Hurtuková and Václav Švorčík

Nowadays, new materials for the preparation of synthetic bone grafts are being sought after. Bones consist mainly of collagen fibres and hydroxyapatite crystals, so using synthetic grafts with a similar structure is logical. Therefore, a composite material was prepared by adding hydroxyapatite particles in the cultivation medium of bacterial nanocellulose (*in-situ* method). The composites were dried via air-drying and lyophilization to obtain solid materials. The formation of the composite was confirmed by infrared spectroscopy, which showed cellulose (OH vibrations) as well as hydroxyapatite ( $\text{PO}_4^{3-}$  vibrations) absorption bands. Energy dispersive X-ray spectroscopy showed presence of 14 wt% of calcium and 48 wt% of phosphorus in the composites. The structure of the hydroxyapatite crystals embedded in fibrous cellulose was observed by scanning electron microscopy. The higher porosity of the lyophilized samples was confirmed as well. Water contact angle of the air-dried composites was lower ( $25 \pm 3.6^\circ$ ) compared to pure bacterial nanocellulose ( $30 \pm 2.7^\circ$ ). The mechanical strength of the air-dried composites was ( $20.3 \pm 7.9$ ) MPa. These results suggest the prepared material is promising for construction of synthetic bone grafts.

## Introduction

If there is a significant loss of bone mass due to a bone defect, it is necessary to use a bone graft to stimulate bone healing and fill in the bone loss [1]. Autografts and allografts are the first choice, but they are limited in supply, lead to associated surgeries, and carry a risk of infection and rejection. Therefore, the search for functional fully synthetic grafts gains on importance [2].

Synthetic bone grafts should be biocompatible, hypoallergenic, non-irritating, osteoinductive (stimulate the stem cells to develop into bone cells), osteoconductive (permit blood vessels and other cells into the structure), and structurally and mechanically similar to bone. Until today, several types of synthetic bone grafts with different compositions and structural and mechanical properties have been developed, many of them are commercially available. Search for new materials focuses on improving of their properties, mainly their osteoconductive activity [1].

Bones consist of organic polymers combined with inorganic nanocrystals of calcium phosphates. It is possible to prepare synthetic materials with a similar structure [3]. The organic part is composed of collagen I fibres that provide mechanical strength (the ability to absorb energy

[4] and help the osteoblasts to form the bone structure [5]. The hydroxyapatite (HAp) nanocrystals contribute to the stiffness [4]. The synthetic graft could therefore consist of collagen-HAp material. This composite material was successfully prepared via several techniques [6], but has certain limitations: collagen is difficult to sterilize and due to its animal origin, it is expensive, carries a risk of pathogen transfers, and could cause allergic reactions [7]. Other natural polymers with similar morphology to collagen - e.g. bacterial nanocellulose (BNC) - could therefore prove useful in this application.

BNC is produced by specific genera of bacteria, especially the genus *Komagataeibacter* and its fibres do not exceed 100 nm in diameter. *In vitro* it forms on the surface of the culture media rich in sugars. Thanks to its nanostructure, BNC has high surface area and porosity leading to a strong interaction with liquids, high stiffness, and better mechanical properties compared to plant cellulose [8].

The BNC-HAp composites can be achieved via *ex-situ* (after the formation of BNC) or *in-situ* (directly in the BNC culture media) methods [9].

In this work, we prepared BNC-HAp composite material via *in-situ* bacterial cultivation. Air-drying and lyophilization was used to solidify the prepared composite hydrogels. The formation of the composite was confirmed by infrared spectroscopy as well as energy-dispersive X-ray spectroscopy and X-ray diffraction. We studied the surface morphology, mechanical and swelling properties together with the water contact angle that represents the surface hydrophilicity/hydrophobicity of the prepared composite.

Department of Solid State Engineering, University of Chemistry and Technology Prague, Technická 5, 166 28 Prague 6, Czech Republic

\*Corresponding author:  
E-mail: kutovaa@vscht.cz

DOI: 10.5185/amlett.2022.041708

The novelty of this work lies in comparison of properties of the air-dried and lyophilized composites as well as in evaluation of the mechanical and swelling properties of the materials, which are very important properties for its potential application.

**Table 1.** List of BNC-HAp preparation approaches.

Authors	Method	Drying	Additives
Grande et al. [10]	<i>in-situ</i>	lyophilization, solvent exchange, hot pressing	Carboxymethyl-cellulose - viscosifier
Niamsamp et al. [11]	<i>in-situ</i>	lyophilization	Cellulose nanocrystals
Zimmermann et al. [2]	<i>ex-situ</i>	-	Carboxymethyl-cellulose – binding agent
Wan et al. [12]	<i>ex-situ</i>	lyophilization	Phosphoric acid – binding agent
Saska et al. [13]	<i>ex-situ</i>	Oven (50°C) drying	CaCl <sub>2</sub> and Na <sub>2</sub> HPO <sub>4</sub> – HAp precursors

## Experimental

### Preparation of the BNC-HAp composites

BNC was produced by *Komagataeibacter sucrofermentans* bacterial strain (DSM 15973, Leibniz-Institut, Germany). Cultivation was carried out in a modified Hestrin-Shramm [14] culture medium consisting of sucrose (20 g/L), disodium hydrogen phosphate dodecahydrate (6.8 g/L), special peptone (5 g/L), yeast extract (5 g/L) and citric acid monohydrate (1.3 g/L) at pH = 5.8. Cultivation was carried out statically in an Erlenmeyer flask containing HAp (BCBS8492V, 2g per 100 ml of medium, Sigma-Aldrich, USA) and lasted for at least 21 days at 28 °C. Purification of the prepared composites from cultivation residues was performed by rinsing twice in boiling 0.1 M NaOH and then rinsing at least twice in boiling distilled water. Washed BNC-HAp hydrogels were solidified by air-drying on PTFE foil (AD) or lyophilization (L) (Alpha 3-4 LSCbasic, Christ, Germany) for at least 24 hours.

### Characterization of BNC-HAp composites

The chemical composition of the composites was studied by infrared spectroscopy (FTIR, Nicolet iS5 with iD7 attenuated total reflection accessory with diamond crystal, ThermoFisher, USA). The spectra were obtained as an average from 128 measurement cycles in 600–4000 cm<sup>-1</sup> spectral range, and 1 cm<sup>-1</sup> data interval.

The X-ray diffraction was employed to confirm the successful preparation of the nanocomposite using the X'Pert PRO powder diffractometer (PANalytical, Netherlands) with copper X-ray lamp ( $\lambda_{CuK\alpha 1} = 0.1540598$  nm). Each sample was scanned in 2 $\theta$  range of 4°–60° with a scanning rate of 5°/min in a symmetric Bragg-Brentano geometry.

Surface morphology was observed by scanning electron microscope (SEM) LYRA3 GMU (Tescan, Czech

Republic) at 10 kV acceleration voltage. The elemental composition was determined by energy-dispersive X-ray spectroscopy (EDS, X-MaxN, 20 mm<sup>2</sup> SDD detector, Oxford Instruments, United Kingdom) during the SEM measurements.

The swelling properties are represented by swelling ratios (SR) of phosphate-buffered saline (PBS). Previously weighed samples were immersed in PBS for one hour. Then they were removed, left in a vertical position for dripping for 30 seconds, and weighed again. The SR was calculated using the equation:  $SR = ((m_1 - m_0)/m_0) \cdot 100\%$ , where  $m_0$ ,  $m_1$  stand for weight before and after immersion, respectively.

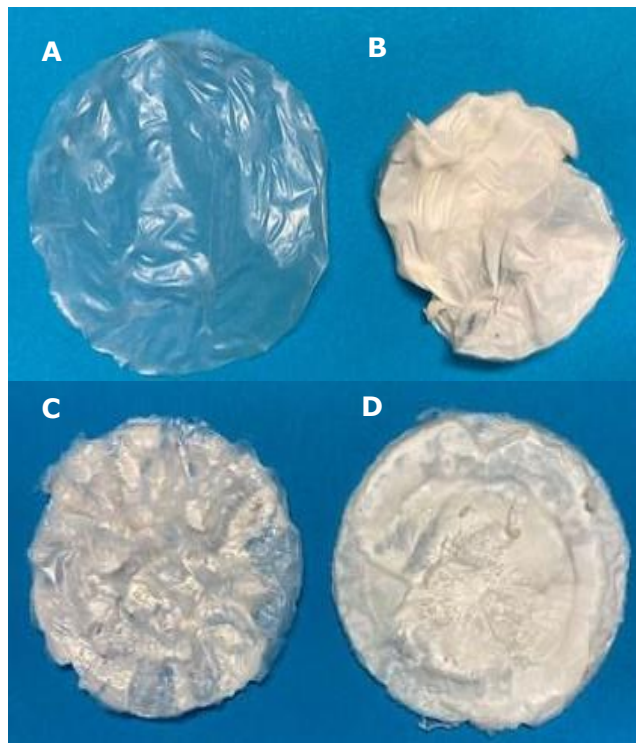
Tensile mechanical properties were measured using a Universal Testing Machine Model 3365 (Instron, USA) according to ISO 37. The dumbbell-shaped samples with the working part 10 mm long and 5 mm wide were cut from dried composite foils. The thickness of the samples was measured with a digital calliper micrometre QuantuMike IP65 (0–25 mm, 0.001 mm, Mitutoyo, Japan). The samples were stretched by 10 mm·min<sup>-1</sup> until fracture. The mechanical properties were calculated from the stress-strain curve that was plotted by the Bluehill software: the tensile strength and tensile strain from fraction moment as the maximum stress and elongation respectively, Young's modulus from the slope of the linear part of the curve.

Water contact angles (WCA) were measured by the sessile drop method by a fully automated goniometer DSA100 (KRÜSS, Germany). Water drops of  $2.0 \pm 0.2 \mu\text{l}$  were deposited on the tested samples, images were taken after a 2 s delay. The contact angle was calculated from the captured image using the height/width method.

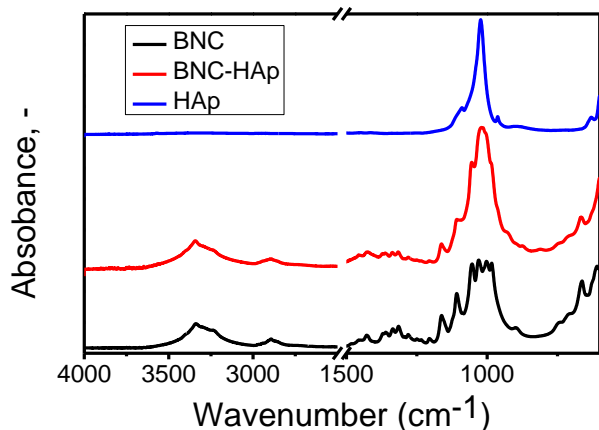
## Results and discussion

The pure BNC and BNC-HAp composite foils prepared by *in-situ* bacterial cultivation are shown in **Fig. 1**. In the case of the AD samples the pure BNC was transparent while the composite material was opaque. This phenomenon was not as apparent for the L samples due to their sponge-like structure [15]. The yields of the composites compared to pure BNC suggest approx. 40 wt% of HAp present in them. The successful composite preparation is confirmed by the FTIR spectra in **Fig. 2**. The single strong absorption band around 1020 cm<sup>-1</sup> in the HAp spectrum corresponds PO<sub>4</sub><sup>3-</sup> vibrations [16]. Absorption bands in the BNC spectrum in this area (950–1200 cm<sup>-1</sup>) are related to C–O stretching modes in hydroxyl groups and with the intra and inter-cyclic C–O vibrations of the acetal groups [17]. BNC has an OH absorption peak around 3350 cm<sup>-1</sup> that significantly distinguishes it from HAp. In the spectrum of BNC-HAp the OH absorption peak around 3350 cm<sup>-1</sup> indicates the presence of BNC and the bands in the 950–1200 cm<sup>-1</sup> range are clearly a combination of BNC and HAp absorption. These are supported by EDS measurements that showed the presence of just C and O in the pure cellulose and the occurrence of 48 wt% of Ca and 14 wt% of P in the

composite samples (note that P bands overlap Pt employed as a coating metal). The inhomogeneous distribution of HAp and BNC in the samples (due to the static cultivation process) means the EDS results cannot be employed to calculate the BNC/HAp ratio in the whole sample of the composite.

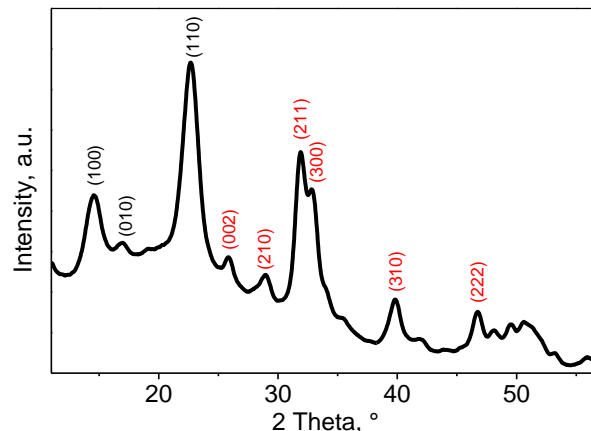


**Fig. 1.** Foils of air-dried BNC (A), air-dried BNC-HAp composite (B), lyophilized BNC (C), and lyophilized BNC-HAp composite (D).



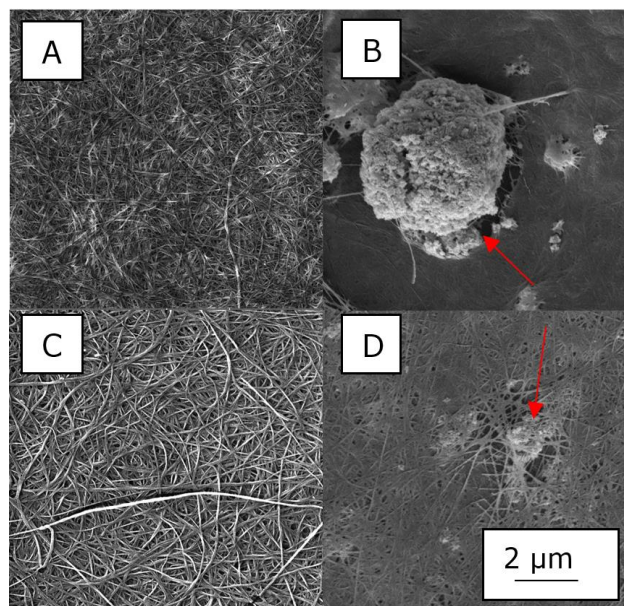
**Fig. 2.** FTIR spectrum of air-dried BNC, air-dried BNC-HAp composite, and pure HAp.

The successful preparation of the composite was confirmed by XRD measurements as well (**Fig. 3**). The diffractogram of the prepared composite shows characteristic peaks for the crystalline planes of both BNC (notable the (100) and (110) planes) and HAp (most notably the (211), (310) and (222) planes) [11,18].



**Fig. 3.** XRD spectrum of air-dried BNC-HAp composite with BNC (in black) and HAp (in red) Miller indices assigned according to [11,18].

The surface morphology of the composites can be observed in the SEM images (**Fig. 4**). The fibrous nanostructure of BNC with crystals of HAp is visible. Compared to the AD samples the L samples show higher porosity in the images.



**Fig. 2.** SEM images of air-dried BNC (A), air-dried BNC-HAp composite (B), lyophilized BNC (C), and lyophilized BNC-HAp composite (D). Arrows show the embedded HAp crystals.

The WCA measurements were employed to study the surface wettability that could be affected by the surface morphology and chemical composition, particularly the amount of hydrophilic/hydrophobic functional groups. The decrease of WCA with introduction of HAp for AD samples was only slight (from  $(30 \pm 2.7)^\circ$  to  $(25 \pm 3.6)^\circ$ ) compared to what was expected based on the literature [19,20]. This could be attributed to the high hydrophilicity of the original BNC. The WCA of L samples were impossible to measure due to their high hydrophilicity and porosity.



The degree to which the samples can absorb PBS is important for biological tests e.g., cell growth and adhesion [21]. We have observed decrease of SR for the composites compared to pure BNC - for AD samples from (930 ± 200)% to (630 ± 130)% and for L samples from (6540 ± 300)% to (2700 ± 300)%. This decrease is probably caused by the clogging of BNC pores with the HAp and therefore is even more pronounced for the more porous L samples [15].

The tensile mechanical properties of our samples are shown in **Table 2**. The AD samples have higher strength as well as elastic modulus compared to L samples. This is caused by the higher porosity of the L samples and by the collapsed nanostructure of the AD samples that leads to higher folding of the fibres and the formation of more hydrogen bonds. The addition of HAp enhances the mechanical properties including the tensile strain at break of AD samples, however, in case of L samples it leads to a decrease in mechanical properties. This could be caused by clogging the BNC pores and disruption of its nanostructure. The measured values are somewhat lower than those of actual human bones (e.g., 90-190 MPa and 2-45 MPa tensile strength for human cortical and cancellous bone, respectively) [22].

**Table 2.** Tensile mechanical properties of BNC and BNC-HAp composites.

	Tensile strength (MPa)	Tensile strain (%)	Elastic modulus (MPa)
Pure BNC AD	16.1 ± 5.8	4.5 ± 0.6	351 ± 174
BNC-HAp AD	20.3 ± 7.9	5.4 ± 1.3	411 ± 109
Pure BNC L	3.5 ± 0.8	8.5 ± 1.9	42 ± 10
BNC-HAp L	1.1 ± 0.4	6.3 ± 1.7	19 ± 7

## Conclusion

Using the *in-situ* cultivation method we prepared the BNC-HAp composite material in a modified Hestrin-Shramm culture medium. Using two methods of drying – air drying and lyophilization – we obtained two different materials with distinct properties. The L samples were more porous and therefore more hydrophilic, but had decreased tensile mechanical properties. Compared to pure BNC, the BNC-HAp composites were more hydrophilic but, surprisingly, they had lower swelling ratio. This could be caused by the clogged pores of the composite material. The mechanical properties were improved for the AD composites, however, they were decreased for the L composites compared to pure BNC. These properties can have significant effect on the cell initial adhesion and subsequent growth. Therefore, the next goal would be to test these composites as bone cells scaffold in tissue engineering.

## Acknowledgements

This work was supported by the GACR under the project 20-16041S and the grant of Specific university research – grant No. A2\_FCHT\_2022\_074.

## Conflicts of interest

There are no conflicts to declare.

**Keywords:** Bacterial nanocellulose, hydroxyapatite, composite.

## References

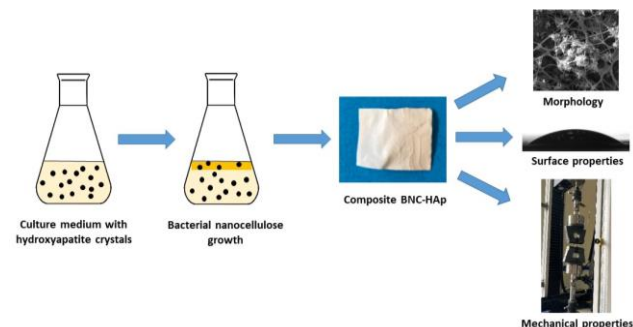
- Giannoudis, P. V.; Dinopoulos, H.; Tsiridis, E.; *Injury*, **2005**, *36*, S20.
- Zimmermann, K. A.; LeBlanc, J. M.; Sheets, K. T.; Fos, R. W.; Gatenholm, P.; *Mat. Sci. Eng. C*, **2011**, *31*, 43.
- Buckwalter, J. A.; Cooper, R. R.; *Instr. Course Lect.*, **1987**, *36*, 27.
- Viguet-Carrin, S.; Garnero, P.; Delmas, P. D.; *Osteoporos Int.*, **2006**, *17*, 319.
- Ferreira, A.M.; Gentile, P.; Chiono, V.; Ciardelli, G.; *Acta Biomater.*, **2012**, *8*, 3191.
- Kołodziejka, B.; Kaflak, A.; Kolmas, J.; *Materials*, **2020**, *13*, 1748.
- Yunus Basha, R.; Sampath Kumar, T. S.; Doble, M.; *Mat. Sci. Eng. C*, **2015**, *57*, 452.
- Klemm, D.; Kramer, F.; Moritz, S.; Lindström, T.; Ankerforst, M.; Gray, D.; Dorris, A.; *Angew. Chem., Int. Ed.*, **2011**, *50*, 5438.
- Shah, J.; Malcolm Brown Jr., R.; *Appl. Microbiol. Biotechnol.*, **2005**, *66*, 352.
- Grande, C. J.; Torres, F. G.; Gomez, C. M.; Bañó, M.C.; *Acta Biomater.*, **2009**, *5*, 1605.
- Niamsap, T.; Lam, N. T.; Sukyai, P.; *Carbohydr. Polym.*, **2019**, *205*, 159.
- Wan, Y. Z.; Hong, L.; Jia, S. R.; Huang, Y.; Zhu, Y.; Wang, Y. L.; Jiang, H. J.; *Compos. Sci. Technol.*, **2006**, *66*, 1825.
- Saska, S.; Barud, H. S.; Gaspar, A. M. M.; Marchetto, R.; Ribeiro, S. J. L.; Messaddeq, Y.; *Int. J. Biomater.*, **2011**, *2011*, 175362.
- Hestrin, S.; Schramm, M.; *Biochem. J.*, **1954**, *58*, 345.
- Kutová, A.; Staňková, L.; Vejvodová, K.; Kvítek, O.; Vokatá, B.; Fajstavr, D.; Kolská, Z.; Brož, A.; Bačáková, L.; Švorčík, V.; *Nanomaterials*, **2021**, *11*, 1916.
- Michelot, A.; Sarda, S.; Audin, C.; Deydier, E.; Manoury, E.; Poli, R.; Rey, C.; *J. Mater. Sci.*, **2015**, *50*, 5746.
- Maréchal, Y.; Chanzy, H.; *J. Mol. Struct.*, **2000**, *523*, 183.
- Lara-Ochoa, S.; Ortega-Lara, W.; Guerrero-Beltrán, C. E.; *Pharmaceutics*, **2021**, *13*, 1642.
- Surmeneva, M.; Nikityuk, P.; Hans, M.; Surmenev, R.; *Materials*, **2016**, *9*, 862.
- Zimina, A.; Senatov, F.; Choudhary, R.; Kolesnikov, E.; Anisimova, N.; Kiselevskiy, M.; Orlova, P.; Strukova, N.; Generalova, M.; Manskikh, V.; Gromov, A.; Karyagina, A.; *Polymers*, **2020**, *12*, 2938.
- Slepičková Kasálková, N.; Slepička, P.; Ivanovská, B.; Trávníčková, M.; Malinský, P.; Macková, A.; Bačáková, L.; Švorčík, V.; *Coatings*, **2020**, *10*, 1083.
- Hannink, G.; Chris Arts, J. J.; *Injury*, **2011**, *42*, S22.

## Authors biography



**Anna Kutová** finished her master studies at UCT Prague in Drug production in 2019. Currently, she is studying Ph.D. at UCT Prague in the field Drugs and Biomaterials. During summer 2019 she was at an internship at Manipal Institute of Technology (MIT) in India. She is an author of one published paper in journal *Nanomaterials*.

## Graphical abstract



Preparation of BNC-HAp composite using the *in-situ* method.