Carbohydrate Polymers 106 (2014) 410–413

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

# Loading of bacterial nanocellulose hydrogels with proteins using a high-speed technique

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ARTICLE INFO

*Article history:*
Received 13 August 2013
Received in revised form
11 December 2013
Accepted 11 January 2014
Available online 21 January 2014

*Keywords:*
Bacterial nanocellulose
Hydrogel
Protein delivery
Controlled release

ABSTRACT

For the loading of the natural biopolymer bacterial nanocellulose (BNC) with drugs, usually an adsorption method has been described. In the present study, a high-speed loading technique based on vortexing was established for the incorporation of proteins in BNC as drug delivery system. Compared to the conventional technique, vortexing accomplished in 10 min the same protein loading capacity as the adsorption method in 24 h with comparable protein distribution and protein stability. Vortex loaded BNC demonstrated a retarded protein release with a lower total amount of released protein after 168 h compared to the adsorption loaded BNC. This was correlated with a densification of the fiber network as shown by electron microscopy and a reduced water holding capacity. These observations offer the possibility to control the drug release by selection of the preparation technique.



## 1. Introduction

The carbohydrate polymer bacterial nanocellulose (BNC) which consists of β-1,4-linked glucopyranose units is an innovative biofabricated material synthesized by *Komagataeibacter* strains as an inherently stable hydrogel with a three-dimensional network of nanofibers (Bielecki, Krystynowicz, Turkiewicz, & Kalinowska, 2005; Klemm et al., 2006). Due to its outstanding mechanical and thermal characteristics, high water content, proven biocompatibility and the control of the biotechnological production, it provides an excellent basis for the use as drug delivery system (Klemm et al., 2011; Svensson et al., 2005). Since native bacterial nanocellulose is not biodegradable or excretable in the human body due to the absence of cellulases, applications such as implants for cartilage replacement, temporary skin, wound dressings, connective-tissue replacements as well as scaffolds for tissue engineering, and artificial blood vessels have become of high interest during the last years (Czaja, Young, Kawecki, & Brown, 2006; Klemm et al., 2006; Petersen & Gatenholm, 2011). However, up to now only a limited number of reports are available describing the incorporation of pharmaceutically active drugs into the nanocellulose network.

Active compounds can be integrated into BNC either during gel formation (in situ) or afterwards in a *post* synthesis modification (Berndt, Wesarg, Wiegand, Kralisch, & Müller, 2013; Kralisch, Hessler, Klemm, Erdmann, & Schmidt, 2010). Typically, most of the drugs like polyhexanide (Wiegand, Abel, Ruth, & Hipler, 2009), lidocain (Trovatti et al., 2011), ibuprofen (Trovatti et al., 2012), vancomycin (Mori, Nakai, Enomoto, Uchio, & Yoshino, 2011) or gentamicin (Mori et al., 2011) were incorporated after the BNC synthesis by a sorption technique, having the advantage of mild conditions and avoiding drug damage during fleece synthesis and purification or impairment of the bacterial growth. Native, semi-dried or dried as well as pressed BNC fleeces were therefore usually immersed in aqueous solutions of the drugs for several hours or days which accomplishes drug loading by diffusional and/or swelling controlled processes. However, this technique is not only time-consuming especially with the view to a commercial production, but might also suffer from stress and instabilities of sensitive drugs.

Therefore in the present study, a fast high-speed technique was established as an alternative to the conventional adsorption loading technique and investigated regarding drug uptake capacity, in vitro release behavior, drug distribution and drug stability. Bovine serum albumin (BSA) was used as a model drug for proteins (Hu et al., 2006) to compare the newly developed vortex technique to the conventional method.

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0144-8617/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2014.01.038

## 2. Materials and methods

### 2.1. Biosynthesis of BNC

BNC fleeces were produced by static cultivation of *Komagataeibacter xylinus* strain DSM 14666 (culture collection of the Friedrich-Schiller-University Jena, deposited at the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in 24-well format and purified as described previously (Müller et al., 2013).

### 2.2. Protein loading and release of BNC

Bovine serum albumin (BSA, fraction V, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was incorporated into BNC by immersion of each sample in 10.0 mL phosphate buffered solution (PBS, Carl Roth) pH 7.4 containing 10 mg/mL (w/v) BSA preserved with 0.03% benzalkonium chloride (Fagron GmbH & Co. KG, Barsbüttel, Germany). BNC fleeces incubated in PBS pH 7.4 without BSA were used as negative controls. Standard sorption loading was performed by incubation under shaking (70 rpm) at 22 °C for 24 h (Müller et al., 2013). High-speed loading was accomplished by vortexing (level 8–9, Vortex®-Genie 2, Scientific Industries Inc., Bohemia, New York, USA) at ambient temperature for 10 min using a tube holder device. For release experiments, samples were transferred each to 20.0 mL PBS pH 7.4. Aliquots of the supernatants of loading and release media were collected at specified time points as indicated for protein quantification by bicinchoninic (BCA) assay (Pierce BCA protein assay kit, Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's instructions. The difference between the amount of BSA in the loading solution before and after loading represented the loaded amount of BSA and the uptake capacity (loaded BSA as percentage of initial amount in the loading solution). Cumulative protein release was calculated based on measured protein amount in aliquots of release media. All experiments were run in triplicate and were repeated once.

### 2.3. Characterization of protein loaded BNC

To visualize the BSA distribution in the BNC compared to controls, loaded samples were incubated in 4 mL BCA assay reagent at 37 °C for 30 min. Photographs were taken in macro mode (Digital camera FinePix E550, Fujifilm Corporation, Tokyo, Japan).

Lyophilized cross sections of BNC samples were sputter-coated with a gold layer at 15 mA in argon atmosphere (S150B, Edwards, Crawley, West Sussex, UK) for scanning electron microscopy (SEM). Micrographs were recorded in vacuo at an acceleration voltage of 20 kV (S440i, Leica Microsystems GmbH, Wetzlar, Germany). Energy dispersive X-ray spectroscopy (EDX) was performed for element analysis using the software Link Isis (Oxford Instruments, Oxfordshire, UK). BNC fiber diameters and pore areas were determined from the micrographs (Axio Vision, Carl Zeiss, Jena, Germany) as described before (Müller et al., 2013).

Aliquots (20 μL) of the release media were electrophoresed (Mini-PROTEAN 3 Cell, Bio-Rad Laboratories GmbH, Munich, Germany) on a 10% polyacrylamide gel (Rotiphorese Gel 30, Carl Roth) in Tris–glycine buffer pH 8.3 (Carl Roth) at a constant voltage of 200 V (Power Pac 1000, Bio-Rad) for native polyacrylamide gel electrophoresis (PAGE). Gel staining was performed with colloidal Coomassie Blue (Roti-Blue, Carl Roth) followed by de-staining with methanol/water and photographing using a gel documentation system (Intas Science Imaging Instruments GmbH, Goettingen, Germany).

For determination of the water holding capacity (WHC), excess liquid was removed from the surface of the samples ($n = 6$) and the loaded fleeces were weighted in closed vials. After air-drying at 80 °C for 24 h the WHC ($g_{water}/g_{cellulose}$) was calculated as the mass of water removed during drying ($g_{water}$) divided by the dry weight of cellulose ($g_{cellulose}$) (Schrecker & Gostomski, 2005).

## 3. Results and discussion

BNC was cultivated as disk shaped hydrogel fleeces characterized by a diameter of 16 mm and a mean weight of 1.2 ± 0.1 g. BSA loading was performed by the conventional technique under gentle stirring over 24 h based on physical adsorption of the protein driven by diffusion along a concentration gradient. Additionally, capillary forces induced by the high hydrophilicity and the large surface area of the BNC fiber network caused by the presence of pore structures and tunnels within the wet BNC fleece are discussed as reason for the uptake of post synthetic processing agents (White & Brown, 1989). The principles of interactions between proteins and the bacterial nanocellulose matrix were already described more detailed by different groups (Andrade, Costa, Domingues, Soares, & Gama, 2010; Bodin et al., 2007; Müller et al., 2013; Ougiya, Hioki, et al., 1998). Within 24 h an uptake capacity of 7.9 ± 0.7% could be achieved (Fig. 1). This method was compared to a newly developed vortex technique that accomplishes the incorporation of the protein into the BNC by dynamic turbulent flow, strong pressure gradients and shear forces which cause a forced but still ordered convection in the flowing medium (Bernard, Thomas, & Handler, 1993). Due to the accelerated influx of protein into the hydrogel, a comparable uptake capacity of 8.4 ± 1.0% could be achieved by the vortex method already within a shorter loading time of 10 min compared to the adsorption method (Fig. 1). The loading capacity was found to be comparable to that discussed for BNC and other non-charged hydrogels before (Gehrke, Uhden, & McBride, 1998; Guemuesderelioglu & Kesgin, 2005; Müller et al., 2013; Schillemans, Verheyen, Barendregt, Hennink, & Van, 2011).

The cumulative release exhibited for both types of loaded BNC a time-dependent biphasic release profile represented by an exponential curve with a rapid release in the initial 6–8 h followed by a slower release rate up to 72 h (Fig. 2). Differences between both methods could be observed regarding velocity and extent of protein release. Although both types of samples showed a similar release rate up to 8 h, adsorption loaded samples demonstrated a faster release and higher total amount of released protein after 24–72 h (conventional adsorption: 91.2 ± 1.9% vs. high-speed loading: 62.4 ± 0.9% of released protein after 72 h). An extension of the release period to 168 h slightly increased the cumulative amount of released BSA to 96.3% and 64.0% for conventionally and high speed loaded BNC, respectively (data not shown).

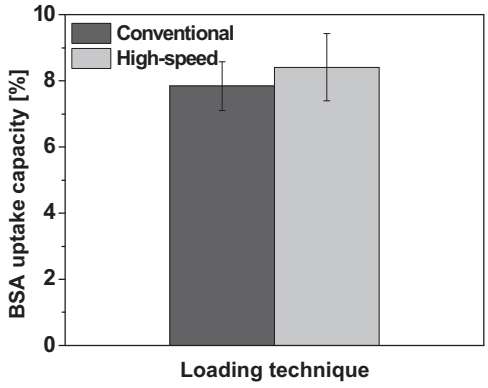


**Fig. 1.** Comparison of BSA uptake capacity (loading as percentage of the loaded amount of BSA in the loading solution) after 24 h conventional adsorption and 10 min high-speed loading (mean ± SD, $n = 3$).