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).

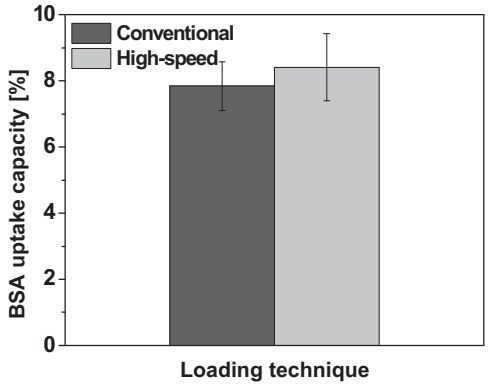

Fig. 1. Comparison of BSA uptake capacity (loading as percentage of the loaded amount of BSA in the loading solution) after 24 h conventional adsorption and 10 min high-speed loading (mean ± SD, $n = 3$).

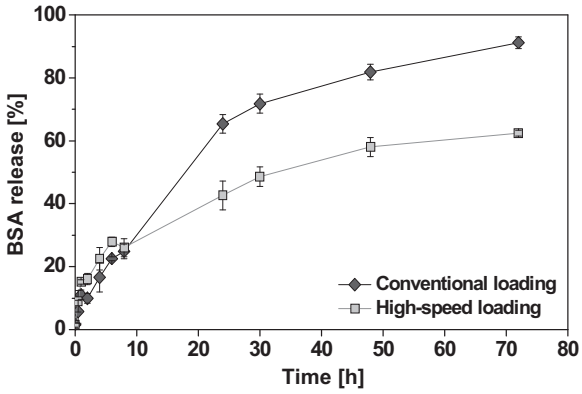

Fig. 2. Cumulative drug release after loading with BSA (10 mg/mL) as a function of time and loading technique (mean ± SD, $n = 3$).

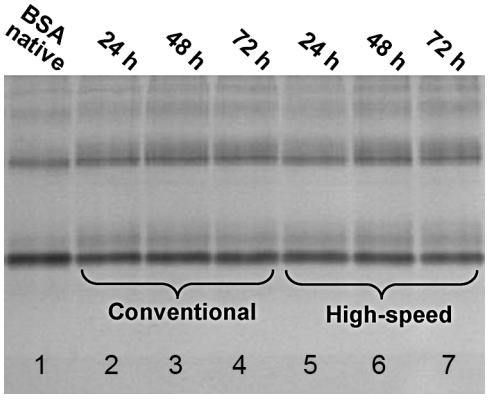

Fig. 3. Time dependent protein stability testing by native polyacrylamide gel electrophoresis after 24, 48 and 72 h: native BSA (lane 1); BSA released after conventional loading (lanes 2–4) and high-speed loading (lanes 5–7).

To exclude differences of the release due to protein instabilities, e.g. due to the high shear stress by vortexing, the protein stability was investigated by native PAGE as shown in Fig. 3. Native BSA displayed two large bands representing monomer and dimer of the protein (Fig. 3, lane 1). Neither degradation to low molecular weight fragments nor formation of high molecular weight aggregates could be observed after 24, 48 and 72 h of release for both sample treatments. Protein stability could therefore be maintained throughout the whole process using the conventional (Fig. 3, lanes 2–4) as well as the high-speed (Fig. 3, lanes 5–7) method. Additionally, macroscopic visualization of the BSA distribution in the BNC hydrogels by BCA staining did not reveal any difference between adsorption (Fig. 4a) and vortex (Fig. 4b) loaded fleeces. Compared to unloaded BNC controls incubated only in PBS pH 7.4 without BSA by adsorption (Fig. 4c) or vortex treatment (Fig. 4d), the cross sections of both types of protein loaded fleeces were homogenously dark-stained with comparable color intensity indicating a homogenous drug distribution in contrast to untreated, unstained controls (Fig. 4e and f).

As protein stability and distribution could not explain the different release characteristics, a deeper insight into the BNC network structure was gained by SEM (Fig. 5). Micrographs of the BNC cross sections demonstrated differences in fiber thickness and network densification dependent on the sample treatment. Untreated BNC control samples showed the typical nanoscaled BNC fiber network and pore system (Fig. 5a). Conventionally loaded samples were characterized by only a slight increase of fiber thickness and pore size (Fig. 5b). A tendency of an increase in the size of pore areas due to further swelling processes during the loading procedure was described before at comparable loading conditions (Müller et al., 2013). In contrast, for the vortex treated BNC a densification of the network structure could be observed as displayed by a pronounced increase in fiber thickness resulting in partial pore closure and reduced fiber distances due to the assembly of adjacent fibers (Fig. 5c). These microscopic observations could also be confirmed by Axio Vision measurements (data not shown). The increased density might result in more resistance from the nanocellulose fibers for protein transport and consequently, may explain the slower release of the BSA from the vortexed BNC. This effect was also described by Huang et al. (2013) for berberin modified BNC, who observed a slower drug release due to fiber swelling and smaller space between BNC fibers. White agglomerate-spots, probably formed during lyophilization before SEM, were qualitatively verified by EDX analysis as buffer salt components (data not shown).

Additionally, the water holding capacity was found to be different for both sample types. Adsorption loaded samples ($91.3 \pm 5.4\, g_{water}/g_{cellulose}$) displayed comparable WHC values as

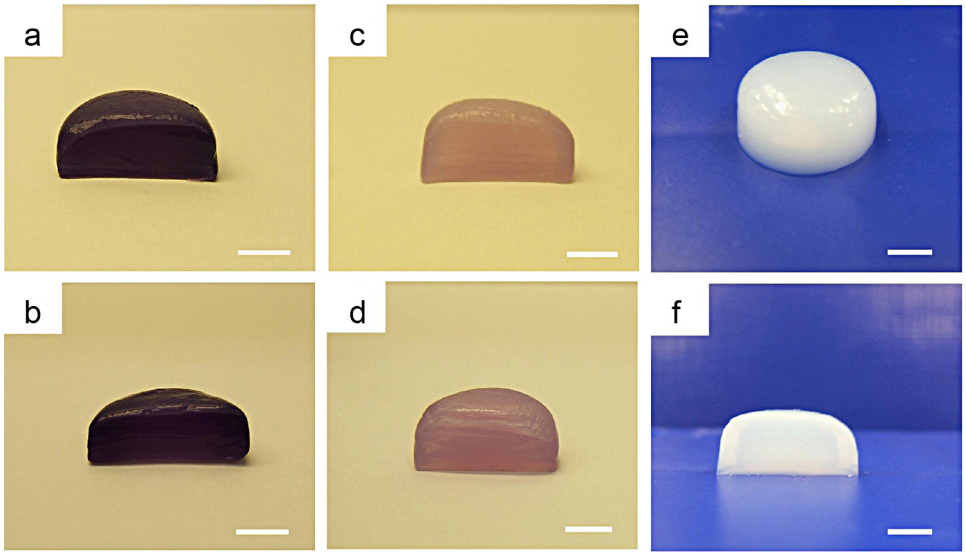

Fig. 4. Visualization of BSA distribution in BNC (cross sections) by staining with BCA assay reagent. BNC loaded by adsorption (a) and high-speed technique (b), corresponding unloaded controls incubated only in buffer without BSA (adsorption, c; vortex treatment, d), and untreated, unstained BNC (e and f) (scale bars, 5 mm).

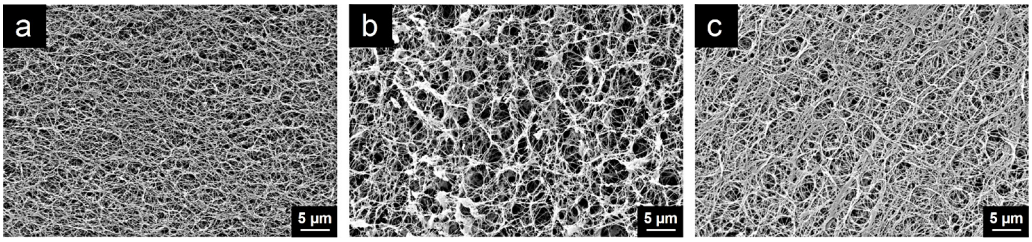

Fig. 5. SEM micrographs (magnification: 1000×) of cross sections of untreated BNC (a) and BNC treated by conventional (b) and vortex technique (c).

the negative control ($97.6 \pm 6.2\, g_{water}/g_{cellulose}$). In contrast, the water holding capacity of the high-speed loaded BNC was found to be strongly reduced ($72.9 \pm 4.6\, g_{water}/g_{cellulose}$) and reached only 75% of the WHC value of the negative control, which indicated structural changes of the BNC network. BNC is naturally characterized by a high water holding capacity due to its high surface area with numerous pores and tunnels (Shezad, Khan, Khan, & Park, 2010). The influence of changes of the BNC structure on the water holding capacity was shown by different groups (Hessler & Klemm, 2009; Watanabe, Tabuchi, Morinaga, & Yoshinaga, 1998). Ougiya, Watanabe, Matsumura, and Yoshinaga (1998) found an increase in WHC of disintegrated bacterial cellulose accompanied with a decreased fibril width. This confirmed our results regarding the correlation of WHC values and fiber thickness.

4. Conclusion

Taking our observations together, the comparison of different post modification techniques revealed that it is possible to load proteins to BNC by a high-speed loading technique based on vortex effects. The technique is now under intensive investigation for its suitability for controllable drug release together with optimization of drug loading capacity and mechanical as well as biological characterizations.

Acknowledgements

Funding by the Thuringian Ministry of Education, Science and Culture as well as the European Fund for Regional Development (B714-10032) is gratefully acknowledged. The authors would like to thank Ramona Brabetz and Elena Pfaff for their excellent technical assistance.

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