

1 **Hydrophobin-nanofibrillated cellulose stabilized emulsions for encapsulation and release of**  
2 **BCS class II drugs**

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11

12 **ABSTRACT**

13 The purpose of this study was to construct biopolymer-based oil-in-water emulsion formulations for  
14 encapsulation and release of poorly water soluble model compounds naproxen and ibuprofen. Class  
15 II hydrophobin protein HFBII from *Trichoderma reesei* was used as a surfactant to stabilize the  
16 oil/water interfaces of the emulsion droplets in the continuous aqueous phase. Nanofibrillated  
17 cellulose (NFC) was used as a viscosity modifier to further stabilize the emulsions and encapsulate  
18 protein coated oil droplets in NFC fiber network. In our previous study it has been shown that  
19 hydrophobin proteins and NFC can be used successfully to stabilize oil-in-water emulsions (Varjonen  
20 et al., *Soft Matter* 7, 2011, 2402-2411). In this study the use of these materials has been extended for  
21 stabilization of pharmaceutical emulsions. The potential of both native and oxidized NFC were  
22 studied for this purpose. Various emulsion formulations were prepared and the abilities of different  
23 formulations to control the drug release rate of naproxen and ibuprofen, used as model compounds,  
24 were evaluated. The optimal formulation for sustained drug release consisted of 0.01% of drug, 0.1%  
25 HFBII, 0.15% oxidized NFC, 10% soybean oil and 90% water phase. By comparison, the use of  
26 native NFC in combination with HFBII resulted in an immediate drug release for both of the  
27 compounds. The results indicate that these NFC originated biopolymers are suitable for  
28 pharmaceutical emulsion formulations. The native and oxidized NFC grades can be used as emulsion  
29 stabilizers in sustained and immediate drug release applications. Furthermore, stabilization of the  
30 emulsions was achieved with low concentrations of both HFBII and NFC, which may be an advantage  
31 when compared to surfactant concentrations of conventional excipients traditionally used in  
32 pharmaceutical emulsion formulations.

33 **Keywords**

1 Nanofibrillated cellulose; Hydrophobin; Emulsions; Drug release; Ibuprofen; Naproxen

## 2 **Abbreviations**

3 **BC**, bacterial cellulose; **BCS**, biopharmaceutical classification system; **DLS**, dynamic light  
4 scattering; **GIT**, gastrointestinal tract; **HFBII**, class II hydrophobin protein HFBII; **IBU**, ibuprofen;  
5 **NAP**, naproxen; **NFC**, nanofibrillated cellulose; **NFC-N**, native nanofibrillated cellulose; **NFC-O**,  
6 TEMPO oxidized nanofibrillated cellulose; **PDI**, polydispersity index; **pI**, isoelectric point

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## 8 **1. Introduction**

9 Oral administration route is the preferred drug delivery route for chronic therapy due to its high patient  
10 compliance and ease of administration when compared to the parenteral routes. The bioavailability  
11 of orally administered poorly water soluble biopharmaceutical classification system (BCS) class II  
12 drugs is limited by their solubility in the varying conditions of the gastrointestinal tract (GIT) (Khadra  
13 et al., 2015). Consequently this may result in poor bioavailability. Several formulation strategies have  
14 been developed to tackle the solubility limitation including soluble complexes, emulsions and self-  
15 emulsifying systems (Fahr, 2007;Singh et al., 2011). In lipid based drug delivery systems the drug  
16 molecules are solubilized in the formulation, which is beneficial for consistent bioavailability of  
17 poorly water soluble drugs. Emulsion formulation strategies have been used to increase the aqueous  
18 solubility of poorly soluble drugs and to control the required bioavailability of these drugs (Gao et  
19 al., 1998;Gorain et al., 2014;He et al., 2011).

20 Emulsions are widely used as pharmaceutical dosage forms as the emulsified materials may obtain  
21 advantages such as improved bioavailability and masking of poor organoleptic properties when  
22 compared to other dosage forms. Typically pharmaceutical emulsions are used for oral, topical or  
23 intravenous administration routes. However, emulsions are thermodynamically unstable and  
24 therefore must be stabilized by addition of an emulsifying agent. Several emulsifying agents can be  
25 used depending on the type of emulsion that is produced (Strickley, 2004). The use of traditional  
26 pharmaceutical emulsifiers may be limited by their concentration dependent toxicity (Davis et al.,  
27 1970;Gloxhuber, 1974). In general, orally administered nonionic and anionic surfactants are low in  
28 toxicity but compatibility with skin and mucous membranes is concentration dependent (Gloxhuber,  
29 1974).

30 Typically used nonionic surfactants in pharmaceutical dosage forms include Tweens, Spans,  
31 Cremphors and Pluronic block copolymers. However, these compounds may interfere with the

1 absorption of certain drugs due to the inhibition of various efflux pumps and transporters present in  
2 the GIT (Rege et al., 2001;Rege et al., 2002;Woodcock et al., 1992). The drug absorption may be  
3 increased if the surfactants affect the functionality of efflux pumps. This may affect the desired  
4 bioavailability levels of certain drug compounds that are substrates for efflux pumps or transporters  
5 in the GIT. In food industry proteins in addition to emulsifiers are widely used in emulsion  
6 stabilization (Wilde et al., 2004). Proteins stabilize emulsions due to their interfacial properties and  
7 the ability to form viscoelastic layers at interfaces. Natural biopolymers, such as proteins, offer an  
8 alternative to synthetic excipients used in the stabilization of pharmaceutical emulsions.

9 Development of nanotechnology has enabled the production of nanocellulose from the natural  
10 polymer cellulose. Nanocellulose term applies to nanofibrillated cellulose (NFC), bacterial cellulose  
11 (BC) and cellulose nanocrystals (Klemm et al., 2011). Plant materials such as wood pulp are  
12 commonly used starting materials for NFC preparation. Typically the production includes mechanical  
13 fibrillation of native cellulose before and/or after enzymatic or chemical pretreatment. The diameter  
14 of native NFC (NFC-N) fibers is typically 5-60 nm and the length is several micrometers. The NFC-  
15 N surface chemistry, hemicellulose content, fiber dimensions and the proportions of crystalline and  
16 amorphous regions may vary depending on the origin of cellulose as well as nanofibrillation  
17 processing (Klemm et al., 2011;Lin and Dufresne, 2014). NFC-N from plant materials may have  
18 hemicelluloses and lignin as accompanying impurities on the surface of the fibrils (Fall et al., 2011).  
19 Hemicellulose contains carboxyl groups and on the surface of NFC-N it introduces a surface charge  
20 to the NFC fibers due to the deprotonation of carboxyl groups.

21 Cellulose can be generally considered to be biocompatible due to moderate, if any, foreign responses  
22 in vivo (Mårtson et al., 1999;Miyamoto et al., 1989). Additionally, NFC has been shown to be  
23 nontoxic in various *in vitro* cell models (Hannukainen et al., 2012;Hua et al., 2014;Vartiainen et al.,  
24 2011). Plant derived NFC-N and BC have been used in various biomedical applications such as drug  
25 delivery, skin tissue repair and tissue bioscaffolds for cellular culture (Kolakovic et al.,  
26 2012;Kolakovic et al., 2013;Malinen et al., 2014;Rouabhia et al., 2014). Plant derived native NFC-N  
27 is a promising matrix material for controlled drug release applications due to its high surface area to  
28 its volume ratio and the multiple carboxyl binding sites on the surface of NFC-N fibers that can  
29 interact with drug molecules via electrostatic interactions (Kolakovic et al., 2013). The electrostatic  
30 binding of molecules with carboxyl groups present on the surface of NFC-N is pH dependent.  
31 Electrostatic interactions between negatively charged carboxyl groups present in NFC-N and  
32 positively charged drugs has been seen as the main binding mechanism for these drugs (Kolakovic et  
33 al., 2013). However, drug interactions with NFC driven by other mechanisms such as aromatic  $\pi$

1 stacking and hydrogen bonding might still take place regardless of the charge of the molecule. The  
2 surface of NFC can be activated via TEMPO [(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl] oxidation to  
3 contain negatively charged carboxyl groups as binding sites for various macromolecules such as  
4 peptides and enzymes (Weishaupt et al., 2015). This approach enables the immobilization of drugs  
5 and macromolecules by electrostatic interactions due to the high density of negative charges on the  
6 surface of TEMPO oxidized NFC (NFC-O). The NFC-O fibers have typically 3-4 nm diameter and  
7 the length in the range of few micrometers (Saito et al., 2006;Saito et al., 2007). Furthermore, NFC-  
8 O has been shown to be nontoxic in *in vitro* cell models (Alexandrescu et al., 2013;Hua et al., 2014).

9 Hydrophobin proteins (HFBs) are a group of small about 10 kDa amphiphilic fungal proteins that are  
10 capable of lowering the interfacial tension by self-assembly into monolayers at  
11 hydrophobic/hydrophilic interfaces such as air-water (a/w) and oil-water (o/w) (Linder et al.,  
12 2005;Linder, 2009;Paananen et al., 2013). Due to this property HFBs have the ability to convert  
13 hydrophobic surfaces into hydrophilic ones and vice versa (Lumsdon et al., 2005;Szilvay et al., 2007).  
14 HFBs are nontoxic by themselves which is a prerequisite for pharmaceutical excipients (Ebbole,  
15 1997). Additionally, a surface layer of HFB on the airborne fungal spores has been shown to prevent  
16 immune recognition (Aimanianda et al., 2009). This is an attractive feature for materials in  
17 pharmaceutical applications. Based on differences in biophysical properties and hydrophathy patterns  
18 HFBs are divided into class I and class II HFBs (Linder, 2009). Class II *Trichoderma reesei* HFBs  
19 (e.g., HFBI, HFBII and HFBIII) are more water soluble than class I HFBs and can be easily dissolved  
20 at rather high concentrations (at least 100 mg/ml) in aqueous solutions (Kisko et al., 2008;Linder et  
21 al., 2005). The amphiphilic nature of HFBs is attributed to the existence of a hydrophobic patch on  
22 the surface of otherwise fairly hydrophilic surface of HFB monomers (Linder et al., 2005). The  
23 overall structure therefore resembles the structure of surfactants with one hydrophobic and one  
24 hydrophilic part. The amphiphilicity makes HFBs suitable surface coating materials for pure drug  
25 crystals as well as drug carrier materials. HFBs have been successfully used as surfactants,  
26 encapsulating agents for nutraceuticals and coatings for drug nanoparticles (NPs) (Israeli-Lev and  
27 Livney, 2014;Sarparanta et al., 2012;Valo et al., 2010). HFBII coating on the surface of drug NPs  
28 has been shown to introduce mucoadhesive properties to drug NPs in the GIT (Sarparanta et al.,  
29 2012). For poorly water soluble drugs, mucoadhesive formulations are beneficial for improving drug  
30 bioavailability due to the prolonged time at the site of drug absorption.

31 Recently NFC has been used in combination with HFBs to produce emulsions, stabilize drug  
32 nanocrystals and to produce aerogel matrices for HFB coated drug NPs (Valo et al., 2011;Valo et al.,  
33 2013;Varjonen et al., 2011). It has been shown in our previous study that HFBs and NFC can be used

1 successfully to stabilize o/w emulsions (Varjonen et al., 2011). Surface active HFB fusion proteins  
2 that contained cellulose-binding domains from cellulolytic enzymes were used. By combining the  
3 fusion protein HFBs and NFC emulsions that contained tightly packed thin NFC films at the oil/water  
4 interface were obtained. In this study *Trichoderma reesei* HFBII and NFC were used for the  
5 stabilization of pharmaceutical emulsions. Due to the interesting surface properties, low toxicity and  
6 good biocompatibility of both NFC and HFBII, these materials were selected for the production of  
7 o/w emulsion formulations.

8 The emulsions were used for the encapsulation and release of poorly water soluble BCS II drugs  
9 naproxen (NAP) and ibuprofen (IBU). HFBII was used as a surfactant to stabilize the o/w interface  
10 of the emulsions, whereas NFC was added to modify the viscosity of the continuous aqueous phase.  
11 The addition of NFC polymer increases the viscosity of the continuous phase in o/w emulsions as  
12 well as w/o/w emulsions and creates a polymer network that limits drop collision, thus reducing the  
13 coalescence phenomena (Carrillo et al., 2015;Dimic-Misic et al., 2013;Garti, 1999;Xhanari et al.,  
14 2011). Furthermore, the bending of hydrophilic NFC at the o/w interface favors positive curvature  
15 towards the organic oil-phase and results in water continuous o/w emulsions (Binks, 2002). These  
16 properties were exploited for emulsion stabilization. The effect of the amount of NFC, HFBII and  
17 their combinations on the emulsion stability was studied. After the preliminary formulation  
18 optimization, the most suitable formulations were selected for the drug release studies. The two  
19 different grades of NFC, native and oxidized, were expected to induce differences in the drug release  
20 profiles.

## 21 **2. Materials and methods**

### 22 *2.1 Materials*

23 Hydrophobin class II protein HFBII from *Trichoderma reesei* was provided by VTT Technical  
24 Research Centre of Finland, Finland. Native (Growdex<sup>®</sup> 1.73%) and oxidized (0.56%) nanofibrillated  
25 cellulose hydrogels (NFC-N and NFC-O, respectively) were kindly provided by UPM-Kymmene  
26 Corporation, Finland. Cellulose kraft pulp was chemically modified and fibrillated to form NFC  
27 hydrogels (Saito et al., 2006;Saito et al., 2007). Due to birch based raw material the NFC-N used in  
28 this work contained some soluble hemicelluloses. This hemicellulose fraction attributes to the slightly  
29 anionic surface charge of NFC-N. Higher negative charge of NFC-O is generated by the carboxyl  
30 groups directly on the fiber surface (carboxylic acid content 1.02 mmol/g pulp). 5 mg/ml stock  
31 solutions of HFBII and NFC-N were prepared in ultrapure water. NFC-O was used at the provided  
32 concentration of 5.6 mg/ml. Soybean oil (batch no. MKBN8800V) and ethyl oleate (batch no.

1 BCBL9739V) were purchased from Sigma-Aldrich, USA and UK respectively. NAP and IBU were  
2 purchased from Sigma-Aldrich, USA and China respectively. 1 mg/ml stock solutions of IBU and  
3 NAP were prepared in soybean oil. Solubility of IBU and NAP in soybean oil was confirmed by  
4 nephelometry (data not shown) (Nepheloscan Ascent, Labsystems, Finland). Acetonitrile was of  
5 analytical grade, Sigma-Aldrich, Germany. Phosphate salts used in the preparation of 0.1 M pH 7.4  
6 phosphate buffer were of analytical grade, Sigma-Aldrich, Germany. Whatman 0.4 µm pore size  
7 Track-Etch polycarbonate membranes were used as semipermeable membranes in the  
8 diffusion studies, Sigma-Aldrich, Germany.

## 9 *2.2 Methods*

### 10 *2.2.1. Emulsion preparation method*

11 Emulsions were prepared from combinations of ultrapure water, soybean oil, ethyl oleate, HFBII 5  
12 mg/ml stock solution, NFC-N 5 mg/ml stock solution and NFC-O 5.6 mg/ml stock solution. To  
13 prevent aggregation in an aqueous environment, HFBII stock solution was sonicated first for 10  
14 minutes before preparing the emulsions. All formulations were premixed by repeated pipetting in  
15 eppendorf tubes prior to ultrasound-sonication. The final emulsion volume was 1 ml for all emulsion  
16 formulations and the volume of the oil phase was 10%. The ultrasound-sonication based method for  
17 the preparation of emulsions was described in our previous study (Varjonen et al., 2011). The same  
18 method was used in this study with minor modifications. The sonication was performed with a high  
19 intensity ultrasound processor (Sonics, USA) equipped with a 2 mm stepped microprobe (power 750  
20 W, frequency 20 kHz and amplitude 30%). During 4 x 30 seconds of sonication the samples were  
21 kept in an ice bath. After the sonication the samples were homogenized in an ultrasonic bath for 1  
22 hour. For high-shear mixing tests, a process time of 1-4 minutes was used (25 000rpm, Ultra-Turrax  
23 T8, IKA WERKE, Germany). In NAP and IBU formulations the drug was dissolved in soybean oil  
24 in 1 mg/ml concentration prior to the emulsion preparation. Table 1 summarizes the different  
25 formulations and their specific contents.

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1 **Table 1.** Emulsion formulation codes and contents. NAP and IBU containing formulations were  
 2 prepared based on preliminary formulations.

Formulation code	HFBII (mg/ml)	NFC-N (mg/ml)	NFC-O (mg/ml)	Soybean oil (10 %)	Ethyl oleate (10 %)	NAP (mg/ml)	IBU (mg/ml)
C <sub>N</sub> S-1		0.1		x			
C <sub>N</sub> S-2		0.5		x			
C <sub>N</sub> S-3		1		x			
C <sub>N</sub> S-4		1.25		x			
C <sub>N</sub> S-5		1.5		x			
C <sub>N</sub> E-1		0.1			x		
C <sub>N</sub> E-2		0.5			x		
C <sub>N</sub> E-3		1			x		
C <sub>N</sub> E-4		1.25			x		
C <sub>N</sub> E-5		1.5			x		
HS-1	0.1			x			
HS-2	0.5			x			
HS-3	1			x			
HS-4	1.25			x			
HS-5	1.5			x			
HC <sub>N</sub> S-1	1	0.5		x			
HC <sub>N</sub> S-2	1	1		x			
HC <sub>N</sub> S-3	1	1.5		x			
HC <sub>N</sub> E-1	1	0.5			x		
HC <sub>N</sub> E-2	1	1			x		
HC <sub>N</sub> E-3	1	1.5			x		
HC <sub>O</sub> S-1	1		0.5	x			
HC <sub>O</sub> S-2	1		1	x			
HC <sub>O</sub> S-3	1		1.5	x			
HC <sub>O</sub> E-1	1		0.5		x		
HC <sub>O</sub> E-2	1		1		x		
HC <sub>O</sub> E-3	1		1.5		x		
NAP-1	1	1		x		0.1	
NAP-2	1		1	x		0.1	
NAP-3	1	1.5		x		0.1	
NAP-4	1		1.5	x		0.1	
NAP-5	1			x		0.1	
IBU-1	1	1		x			0.1
IBU-2	1		1	x			0.1
IBU-3	1	1.5		x			0.1
IBU-4	1		1.5	x			0.1
IBU-5	1			x			0.1
<b>H =</b> HFBII <b>C<sub>N</sub> =</b> NFC-N (native nanofibrillated cellulose) <b>C<sub>O</sub> =</b> NFC-O (TEMPO oxidized nanofibrillated cellulose) <b>S =</b> soybean oil <b>E =</b> ethyl oleate <b>IBU =</b> ibuprofen formulation <b>NAP =</b> naproxen formulation							

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### 1 2.2.2. *Emulsion droplet size and zeta-potential measurements*

2 HFBII stabilized emulsions were diluted 1:10 with dilute HFBII (0.01 mg/ml in ultrapure water)  
3 solution to avoid the possible breakage of HFBII protein coated emulsion droplet o/w interface  
4 surface structures. The zeta-potential values of emulsion droplets were measured by a ZetaSizer  
5 Nano-ZS (Malvern Ltd., UK) in water at 25°C in a folded capillary cell (Malvern Ltd., UK). Dynamic  
6 light scattering (DLS) measurements for hydrodynamic diameter and size distribution of emulsion  
7 droplets were also measured by a ZetaSizer Nano-ZS (Malvern Ltd., UK) in water at 25°C in  
8 disposable polystyrene cuvettes (Sarstedt, Germany). Size measurements were performed at day 1 and  
9 at day 28 after the emulsion preparation, whereas zeta-potential measurements were performed only  
10 at day 1.

### 11 2.2.3. *Emulsion stability index (ESI)*

12 The stability of the emulsions was evaluated by following the emulsion phase separation. Qualitative  
13 emulsion stability index (ESI) values were used to evaluate the phase separation of emulsions during  
14 1 month storage at 4°C in glass vials (Varjonen et al., 2011). Digital camera images were taken to  
15 follow the phase separation of clear aqueous phase from the opaque emulsion phase. The volume  
16 fraction of opaque emulsion phase was determined from the separation of the clear aqueous phase.  
17 The digital camera images were analyzed with ImageJ software (National Institutes of Health,  
18 Bethesda, USA). With ImageJ, a line was drawn from the top to the bottom of the emulsion in the  
19 glass vial. Maximum and minimum intensities along the line length that represented the total volume  
20 were determined in order to estimate emulsion separation. The distance values in pixels (x-axis) were  
21 plotted against gray values (y-axis). The distance values were normalized to represent the total  
22 emulsion volume (x-axis). The gray values were normalized to represent emulsion/water separation  
23 (y-axis). Above or at a value of 0.5 (at a scale of 0-1) at y-axis, the corresponding volume fraction at  
24 x-axis was considered to be unseparated opaque emulsion. Below the value of 0.5 at y-axis, the  
25 corresponding volume fraction at x-axis was considered to be separated clear aqueous phase. ESI  
26 values were calculated from the volume of separated water and total volume as follows.

$$27 \quad ESI = 1 - \frac{V_{w,separated}}{V_{total}}$$

28 ESI value 1 corresponds to completely unseparated emulsion whereas 0 is a value for completely  
29 separated system. The ESI values were calculated at day 1, 3, 7, 14, 21 and 28 after the emulsion  
30 preparation.

### 31 2.2.4 *Viscosity measurements*



1 The shear rate viscosity of ultrapure water, 0.1 % and 0.15 % NFC-N and NFC-O was measured at  
2 4°C with HAAKE Viscotester iQ Rheometer (Thermo Fisher Scientific, Karlsruhe, Germany)  
3 equipped with a Peltier system for temperature control. Results were analyzed with HAAKE  
4 RheoWin 4.0 software (Thermo Fisher Scientific). Cup and double-gap cylinder geometry was used  
5 with a 4.0 mm gap and 2.64 ml sample volume. Before each measurement, the samples were allowed  
6 to rest for 2 min at 4°C. Shear viscosity was measured by increasing the shear rate from 100 to 1900  
7 1/s. All measurements were performed in triplicate.

#### 8 *2.2.5. Screening of oils for emulsions*

9 The solubility of NAP and IBU in soybean oil and ethyl oleate was measured. An excess amount of  
10 NAP and IBU were added to both oils, and then mixed with magnetic stirring. After stirring for 72 h  
11 at 25 °C, the equilibrated samples were centrifuged for 10 min at 14 000 g to remove excess amount  
12 of undissolved drug. Then the supernatant was filtered through a 0.2 µm pore size Minisart syringe  
13 filter, Sartorius, Germany. Filtered supernatants were diluted first with methanol/acetonitrile (50:50)  
14 and then further with acetonitrile/phosphate buffer pH 2 (40/60). The concentrations of NAP and IBU  
15 were determined with Ultra performance liquid chromatography (UPLC) instrument Acquity UPLC  
16 (Waters, USA).

#### 17 *2.2.4. Cryo-TEM and light microscopy*

18 Optical microscopy images of emulsions were taken with an optical microscope (Leica DMLB,  
19 Germany) and a microscope coupled digital camera as a quality control. Emulsion samples were  
20 diluted 1:10 for light microscopy imaging. A single droplet of an emulsion was dropped between the  
21 object plate and the cover glass for optical imaging. Emulsions were further imaged and characterized  
22 with 200 kV Tecnai F20 Cryo-TEM microscope (FEI Company, Holland). Before freezing, the 300  
23 mesh copper grids (Quantifoil R2/2, Holey Carbon Film) were treated with glow discharge treatment.  
24 The samples were frozen as thin films with automatic plunge freezing and bare grid technique (Leica  
25 EM GP, Germany) using liquid nitrogen cooled ethane.

#### 26 *2.2.5. In vitro drug release studies*

27 *In vitro* drug release studies were performed in side-by-side-diffusion cells (Laborexin, Finland).  
28 Whatman 0.4 µm pore size Nuclepore Track-Etch polycarbonate membrane filters were clamped  
29 between two identical halves of diffusion cells. The exposed surface area of the clamped membrane  
30 was 0.785 cm<sup>2</sup>. The diffusion cells were kept at 37°C by a surrounding water socket to mimic  
31 physiological conditions. 0.8 ml of drug containing emulsion (0.1 mg/ml of drug) was transferred  
32 into the donor compartment, which was filled to 3.5 ml final volume with 0.1 M phosphate buffer pH

1 7.4. The acceptor compartment was filled with 3.5 ml of 0.1 M pH 7.4 phosphate buffer. Free drug  
2 solutions of NAP and IBU were used as drug release controls through the membrane. The control  
3 samples in the donor compartment contained 0.08 mg of drug per 3.5 ml volume corresponding to  
4 100 % of drug in the emulsions. 200  $\mu$ l samples were collected from the acceptor compartment and  
5 replaced with 200  $\mu$ l of fresh buffer at 0.17, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h. The free drug solutions of  
6 NAP and IBU were used as controls.

7 NAP and IBU concentrations from the *in vitro* drug release samples were analyzed with Ultra  
8 performance liquid chromatography (UPLC) instrument Acquity UPLC (Waters, USA). For NAP,  
9 the column was a BEH Shield column 1.7  $\mu$ m (2.1 x 50 mm) (Waters, USA) at 30°C. The flow rate  
10 was 0.5 ml/min and the injection volume 5  $\mu$ l. The detection was performed at a wavelength of 231  
11 nm. During the gradient run, the mobile phase consisted of a mixture of acetonitrile and 15 mM  
12 phosphate buffer pH 2 in 30:70 ratio at 0 - 3 min. After 3 min, the mobile phase ratio was changed to  
13 70:30. The retention time of NAP was 1.49 min. The linear concentration of NAP was established in  
14 the range of 0.1 - 10  $\mu$ g/ml and LOQ for NAP was 0.007  $\mu$ g/ml. For IBU the column was a HSS-C18  
15 column 1.8  $\mu$ m (2.1 x 50 mm) (Waters, USA) at 30°C. The flow rate was 0.5 ml/min and the injection  
16 volume 5  $\mu$ l. The detection was performed at a wavelength of 219 nm. During the isocratic run the  
17 mobile phase consisted of a mixture of acetonitrile and 15 mM phosphate buffer pH 2 in 60:40 ratio.  
18 The retention time of IBU was 0.85 min. The linear concentration of IBU was established in the range  
19 of 0.1 - 25  $\mu$ g/ml and LOQ for IBU was 0.09  $\mu$ g/ml.

### 20 **3. Results and discussion**

#### 21 *3.1. Emulsion droplet size and zeta-potential measurements*

22 Measurements of emulsion droplet diameters and zeta potential values were performed in order to  
23 estimate the minimum amount of the protein needed to gain stable emulsion droplets with the  
24 sonication method (Varjonen et al., 2011). Sonication produces emulsions with ultrasound-driven  
25 mechanical vibrations. Droplet size, polydispersity index (PDI) and zeta potential measurements were  
26 performed for HFBII stabilized and 10% soybean oil containing preliminary formulations. These HS-  
27 1 to HS-5 formulations contained 0.1–1.5 mg/ml of HFBII as the sole emulsion stabilizer (see Table  
28 1). DLS measurements were not possible with the samples containing NFC as the light scattering by  
29 the long fibers distorted the signal too much.

30 Higher HFBII concentrations in the aqueous phase reduced the emulsion droplet sizes effectively  
31 from 2013 nm to 345 nm and increased the zeta potential values from 25 mV to 32.7 mV (Table 2).  
32 Based on DLS and zeta potential measurements it was concluded that HFBII concentrations between

1 1-1.5 mg/ml (HS-3–HS-5), in the aqueous phase of the emulsions, resulted in good colloidal stability  
 2 at day 1. These formulations had smaller droplet sizes and higher zeta potential values over 30 mV  
 3 when compared to the formulations with smaller amounts of HFBII. However, the PDI values were  
 4 quite high for all formulations indicating that the size distribution of droplets was wide in all  
 5 emulsions. It might be possible to obtain monodisperse emulsion droplets with other techniques like  
 6 homogenization (Maa and Hsu, 1999). At day 28 the emulsion droplet size had increased significantly  
 7 for formulations with low amount of HFBII (0.1-0.5 mg/ml) when compared to day 1 results. The  
 8 amount of HFBII in these emulsions was therefore insufficient. For formulations that contained  
 9 higher amounts of HFBII (1-1.5mg/ml) a decrease in the droplet size at day 28 was observed. This  
 10 might have been caused by the loss of excess amount of protein at the o/w interface. As HFBII is  
 11 known to self-associate and form tetramers in an aqueous solution at the concentration range 0.5-10  
 12 mg/ml (Kisko et al., 2008).

13 **Table 2.** DLS and zeta potential measurements at day 1 and 28 after the preparation (mean  $\pm$  S.D.,  
 14 n=3). Preliminary formulations HS-1 to HS-5 contained 0.1 - 1.5 mg/ml of HFBII as the sole emulsion  
 15 stabilizer. Higher HFBII concentrations reduced the emulsion droplet sizes and increased the zeta  
 16 potential values. PDI values were relatively high for all formulations indicating polydispersity.  
 17 Abbreviations: H=HFBII and S=soybean oil.

Formulation	Day 1: z-average diameter (nm)	Day 28: z-average diameter (nm)	Day 1: PDI	Day 28: PDI	Day 1: Zeta Potential (mV)
HS-1	2013 $\pm$ 33	4623 $\pm$ 1879	0.517 $\pm$ 0.014	1 $\pm$ 0	25 $\pm$ 0.6
HS-2	537.6 $\pm$ 11.7	696.8 $\pm$ 208.4	0.415 $\pm$ 0.028	0.492 $\pm$ 0.219	24.8 $\pm$ 0.2
HS-3	339.0 $\pm$ 1.6	324.9 $\pm$ 39.0	0.440 $\pm$ 0.012	0.349 $\pm$ 0.035	31.4 $\pm$ 1.0
HS-4	301.4 $\pm$ 7.3	194.1 $\pm$ 0.9	0.443 $\pm$ 0.024	0.144 $\pm$ 0.027	30.2 $\pm$ 1.0
HS-5	345.0 $\pm$ 4.7	183.6 $\pm$ 1.3	0.520 $\pm$ 0.017	0.129 $\pm$ 0.006	32.7 $\pm$ 1.1

18

19 HS-3 formulation contained 1 mg/ml of HFBII and the emulsion droplet size did not change  
 20 drastically during 28 days. Therefore, 1 mg/ml HFBII concentration was selected to be used in the  
 21 combination formulations with NFC fibers. HFBII has been shown to adsorb to hydrophobic  
 22 interfaces, such as hydrophobic polymer films and o/w interphase of an emulsion, and to lower the  
 23 interfacial tension even at low concentrations (Lumsdon et al., 2005). 0.2 mg/ml HFBII concentration  
 24 in the aqueous phase has been shown to stabilize o/w emulsions effectively. These results support our  
 25 finding that HFBII can be used as a surfactant to stabilize emulsions at extremely low concentrations.  
 26 Furthermore HFBs are nontoxic, which is an advantage when HFBs are used as pharmaceutical  
 27 excipients (Aimanianda et al., 2009;Ebbole, 1997).

1 The sonication method produced submicron emulsion droplets with high zeta potential values (Table  
 2 2). Therefore, the emulsion quality was considered to be sufficient for the evaluation of HFBII and  
 3 NFC materials in the stabilization of pharmaceutical emulsions. Different sonication times and high-  
 4 shear mixing were tested with HS-3 formulation in order to evaluate further whether the emulsion  
 5 droplet size and PDI values could be decreased. Based on the results it was observed that a sonication  
 6 time of 2 minutes was optimal (Table 3). Longer sonication times or high-shear mixing resulted in  
 7 over processing and significant increase in the droplet size. The size range of droplet diameters was  
 8 in micrometers with high-shear mixing as opposed to nanometers obtained with sonication.  
 9 Therefore, high-shear mixing was not suitable as a preparation process. Furthermore, it was observed  
 10 that it was beneficial to mix all formulation components gently by repeated pipetting prior to  
 11 sonication while preparing the formulations that contained NFC in addition to HFBII. The addition  
 12 of NFC after the sonication of HFBII and other formulation components (oil and water) was tested,  
 13 but this approach led to fast phase separation in a matter of minutes.

14 **Table 3.** The effect of the preparation process on emulsion droplet size and PDI values (mean  $\pm$  S.D.,  
 15 n=3). HS-3-formulation was prepared with sonication and high-shear mixing techniques with  
 16 different mixing durations. Sonication technique produced overall smaller emulsion droplet diameters  
 17 than the high-shear mixing technique.

Preparation method	Day 1: z-average diameter (nm)	Day 28: z-average diameter (nm)	Day 1: PDI	Day 28: PDI
2 min sonication	339.0 $\pm$ 1.6	324.9 $\pm$ 39.0	0.440 $\pm$ 0.012	0.349 $\pm$ 0.035
3 min sonication	415.3 $\pm$ 6.1	366.0 $\pm$ 6.2	0.796 $\pm$ 0.046	0.310 $\pm$ 0.008
4 min sonication	550.0 $\pm$ 18.7	480.0 $\pm$ 11.8	0.937 $\pm$ 0.045	0.540 $\pm$ 0.027
5 min sonication	605.9 $\pm$ 55.3	357.0 $\pm$ 35.0	0.834 $\pm$ 0.113	0.550 $\pm$ 0.090
1 min high-shear mixing	4117 $\pm$ 468	nd	0.319 $\pm$ 0.052	nd
2 min high-shear mixing	4327 $\pm$ 202	nd	0.372 $\pm$ 0.073	nd
4 min high-shear mixing	6388 $\pm$ 120	nd	0.123 $\pm$ 0.029	nd

18

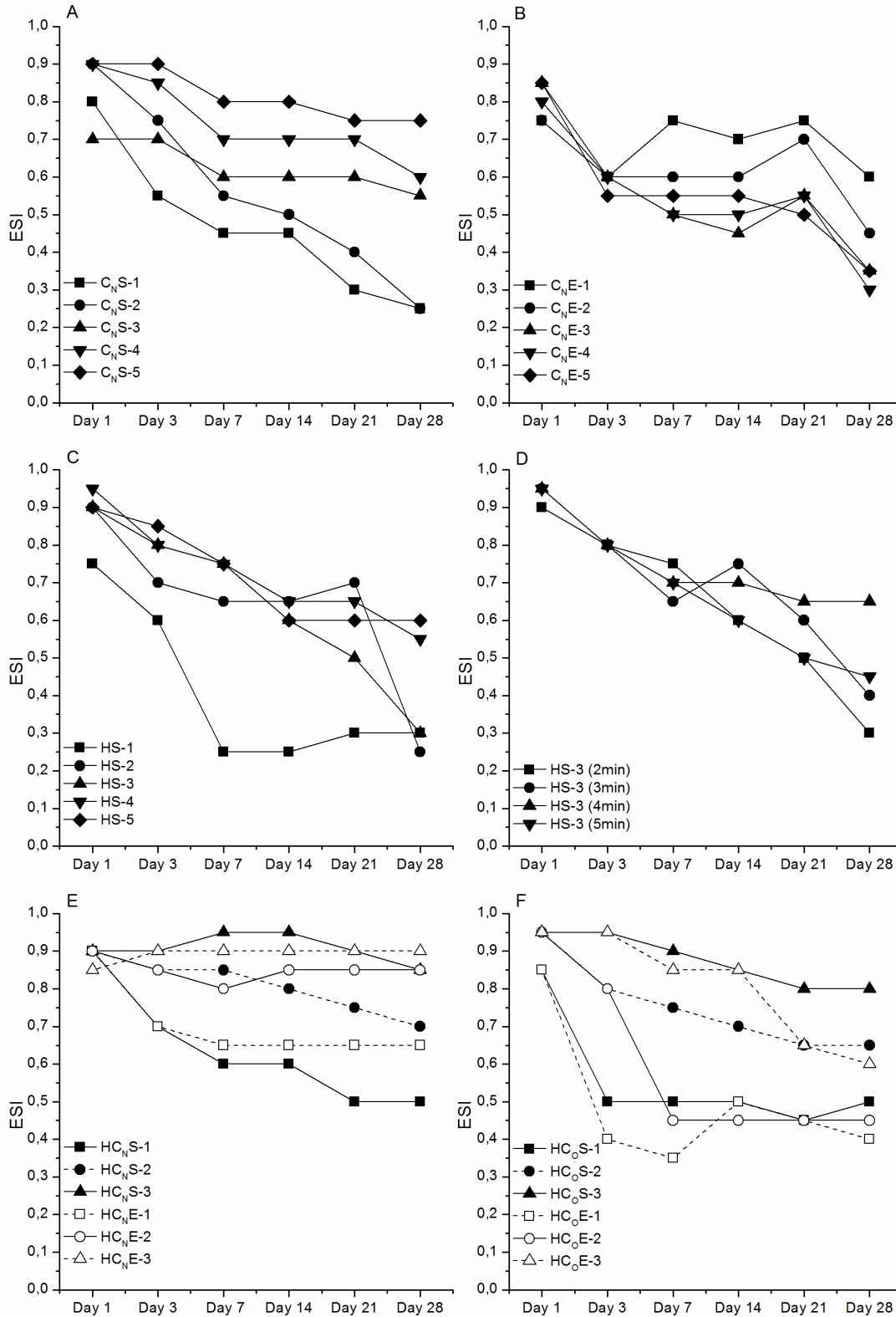
### 19 3.2. NFC and HFBII as emulsion stabilizers

#### 20 3.2.1. Formulations and emulsion stability index (ESI)

21 Formulation optimization process was performed by evaluating the ability of different formulation  
 22 components and their combinations to stabilize the emulsion. Emulsions of soybean oil or ethyl oleate  
 23 and water in the presence of HFBII (1 mg/ml) and/or NFC were studied (see Table 1 for details).  
 24 Soybean oil and ethyl oleate are pharmaceutically acceptable oils that have been used successfully in  
 25 a variety of pharmaceutical formulations (Chen et al., 2006; Cui et al., 2009; Gorain et al., 2014; Wulff-  
 26 Pérez et al., 2009). However, soybean oil has a broader use as it is FDA approved for intravenous,

1 oral and topical formulations. The stability of different formulations were evaluated by following the  
2 phase separation of emulsions with ESI (Fig. 1A-F). The best material combinations were selected  
3 for IBU and NAP formulations based on the highest ESI values of preliminary formulations.

4 The ability of NFC-N (0.1-1.5 mg/ml) to stabilize the emulsions increased when a higher amount of  
5 NFC-N was used in combination with soybean oil (Fig. 1A-B). NFC-N formulations with soybean  
6 oil were more stable than with ethyl oleate during the 28 day period, which could be observed as  
7 higher ESI values and less phase separation. The stability of the soybean oil based emulsions  
8 increased in direct proportion to the amount of NFC-N, whereas the effect of NFC-N amount on the  
9 stability of ethyl oleate emulsions could not be seen as clearly. It has been previously shown that a  
10 continuous NFC-N matrix forms in the aqueous phase of o/w emulsions when NFC-N concentration  
11 is increased to 1 mg/ml (Varjonen et al., 2011). This was reportedly followed by association of oil  
12 droplets to the NFC-N fibrils most likely caused by the emulsification process. Best stability was  
13 gained in our study when at least 1.5 mg/ml NFC-N was used with soybean oil in emulsion  
14 stabilization. Reportedly the addition of NFC increases the viscosity of the continuous phase of o/w  
15 emulsions and creates a polymer network that limits drop collision, thus reducing the coalescence  
16 phenomena (Carrillo et al., 2015;Dimic-Misic et al., 2013;Garti, 1999;Xhanari et al., 2011).  
17 Therefore, the stability of NFC-N containing emulsions depended on the concentration of NFC-N  
18 due to the increased viscosity of the water phase as well as the benefits gained from increasing the  
19 amount of NFC for encapsulation of the oil droplets.



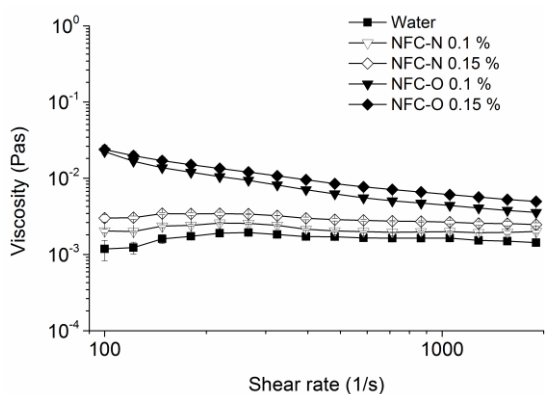
1

2 **Figure 1.** Emulsion stability index (ESI) values for different emulsion formulations (n=1): A) NFC-  
 3 N with soybean oil, B) NFC-N with ethyl oleate, C) HFBII with soybean oil, D) 1 mg/ml HFBII (HS-  
 4 3) with soybean oil prepared by different sonication times, E) HFBII-NFC-N formulations with  
 5 soybean oil or ethyl oleate, F) HFBII-NFC-O formulations with soybean oil or ethyl oleate.  
 6 Abbreviations: H=HFBII, C<sub>N</sub>= NFC-N (native nanofibrillated cellulose), C<sub>O</sub>= NFC-O (TEMPO  
 7 oxidized nanofibrillated cellulose), S=soybean oil and E=ethyl oleate.

1 When HFBII (0.1-1.5 mg/ml) was used for emulsion stabilization only the formulation that contained  
2 the smallest amount of HFBII (0.1 mg/ml) was clearly unstable (Fig. 1C). The most stable  
3 formulations contained 1.25-1.5 mg/ml of HFBII, which indicated clearly that the stability of these  
4 emulsions increased in direct proportion to HFBII content. However, 1 mg/ml HFBII concentration  
5 (HS-3 formulation) was selected to be used in combination with NFC due to the size and PDI  
6 measurements that clearly indicated 1.25 and 1.5 mg/ml concentrations to be excessive (Table 2).  
7 The stability of 1 mg/ml HFBII stabilized HS-3 formulation series that was prepared with different  
8 sonication times was tested (Fig. 1D). Differences in emulsion stability evaluated by phase separation  
9 were quite small. By contrast, the emulsion droplet size and PDI value increased when sonication  
10 time was increased. Regarding the overall emulsion stability 2 min sonication time was optimal.

11 The stability of the emulsions was increased when adequate amounts of HFBII and NFC-N or NFC-  
12 O were used in combination (Fig. 1E-F). The ESI values were higher than when sole emulsion  
13 stabilizers were used in the formulations. We tested 0.5, 1 and 1.5 mg/ml concentrations of NFC-N  
14 and NFC-O in combination with 1 mg/ml HFBII and evaluated the impact of the two oils, soybean  
15 oil and ethyl oleate, on ESI values. Formulations with low amounts (0.5 mg/ml) of NFC-N or NFC-  
16 O proved to be quite unstable as the emulsion phase separation was quite fast already at day 3. The  
17 most stable formulations had 1.5 mg/ml of NFC-N or NFC-O in combination with 1 mg/ml of HFBII.  
18 Relatively stable emulsions were obtained when the surfactants like properties of HFBII were  
19 combined with NFC that can be used to modify the viscosity of the water phase in addition to droplet  
20 encapsulation. Based on the emulsion stability data, the HFBII concentration was kept at a constant  
21 1 mg/ml and two different NFC-N and NFC-O concentrations, 1 mg/ml and 1.5 mg/ml, were selected  
22 for the drug containing formulations in the release studies.

23 Shear viscosities of 1 mg/ml (0.1 %) and 1.5 mg/ml (0.15 %) of NFC-N and NFC-O were measured  
24 as a function of shear rate in order to evaluate the effect of NFC concentration on the viscosity of  
25 aqueous continuous phase in emulsions (Fig. 2). NFC-N at low concentrations of 0.1% and 0.15%  
26 did not affect the viscosity significantly as the shear rate did not affect the viscosity and the viscosity  
27 profile was similar to pure water. NFC-O had approximately ten times higher viscosity values than  
28 NFC-N at 100 (1/s) shear rate. Furthermore, moderate shear thinning was observed for NFC-O. It  
29 has been shown for both NFC grades in several previous studies that the concentration affects the  
30 viscosity and intense shear thinning behavior is observed (Benhamou et al., 2014;Bhattacharya et al.,  
31 2012;Nechyporchuk et al., 2014;Pääkkö et al., 2007). NFC hydrogels can be classified as  
32 pseudoplastic materials in this regard.



1

2 **Figure 2.** Shear rate viscosities of water, 0.1 % and 0.15 % of NFC-N and NFC-O at 4 °C (mean ±  
 3 S.D., n=3).

4 The low shear viscosity of 0.1% and 0.15% NFC-N and NFC-O was not surprising as there was a  
 5 very small amount of fibers to form the NFC network in the aqueous environment. Upon exposing  
 6 increased shear rate the weakly bonded network falls apart easily and individual fibers start to flow.  
 7 However, as NFC-O had slightly higher shear viscosity at 4 °C, which was also used as the storage  
 8 temperature of emulsions, it seems that NFC-O may be more effective in emulsion stabilization due  
 9 to modified viscosity of the continuous phase even at low concentrations. The effect of low NFC-N  
 10 concentration on viscosity was minimal. The emulsion stabilizing effect of NFC-N at 0.1 - 0.15 %  
 11 concentration may be attributed to the reduced emulsion droplet movement rather than the increased  
 12 viscosity of the continuous phase.

13 The solubility of NAP and IBU in soybean oil and ethyl oleate was measured to find the drug  
 14 solubilization capacity of the oils (Table 4). Ethyl oleate had a higher solubilization capacity than  
 15 soybean oil with regard to both drugs. The solubility of NAP was 2.8 g/L in soybean oil and 5.1 g/L  
 16 in ethyl oleate. For IBU, the solubility was 46.2 g/L in soybean oil and 98.7 g/L in ethyl oleate. The  
 17 different drug solubilization capacity of soybean oil and ethyl oleate is attributed to the structural  
 18 differences of these oils (Chen et al., 2006; Date and Nagarsenker, 2008; Sharif Makhmalzadeh et al.,  
 19 2012). Vegetable origin soybean oil contains triglycerides with long hydrocarbon chains and is a high  
 20 molecular volume oil, whereas fatty acid ester ethyl oleate is a lower molecular volume oil than  
 21 soybean oil (Date and Nagarsenker, 2008). Soybean oil with a 1563 Å<sup>3</sup> molecular volume has been  
 22 shown to have a slightly lower solubilizing capacity for lipophilic drugs than ethyl oleate with a 600  
 23 Å<sup>3</sup> molecular volume (Malcolmson et al., 1998). However, the solubility of both drugs in tested oils  
 24 was significantly higher than the aqueous solubility. Therefore, the emulsion formulations containing  
 25 either of these oils can be used to effectively solubilize both NAP and IBU. Soybean oil was selected  
 26 for the oil phase in the emulsions and for the solubilization of poorly water-soluble model drugs NAP



1 and IBU as it proved to be slightly better than ethyl oleate with regard to ESI values. 10% of oil phase  
 2 and 90 % of aqueous phase with 0.1 mg/ml drug loading was used for all emulsions. The drug amount  
 3 in the emulsions can be improved by a higher amount of drug in the oil phase or by a higher amount  
 4 of the oil phase. However, these conditions were chosen for the release studies in order to evaluate  
 5 the performance of the emulsion stabilizing excipients. Further studies are needed to optimize the  
 6 amount of NFC and HFBII for emulsions with a higher volume of oil phase.

7 **Table 4.** Solubility values for NAP and IBU in aqueous media, soybean oil and ethyl oleate.

Compound	Molar mass (g/mol)	pKa	logP	Solubility in aqueous media	Solubility in soybean oil	Solubility in ethyl oleate
NAP	230.3	4.15	3.18	15.9 mg/L, MQ <sup>a</sup> 2.8 - 3.6 g/L, pH=7.4 <sup>b</sup>	2.8 g/L	5.1 g/L
IBU	206.3	4.85	3.97	21 mg/L, MQ <sup>c</sup> 6 g/L, pH=7.4 <sup>d,e</sup>	46.2 g/L	98.7 g/L

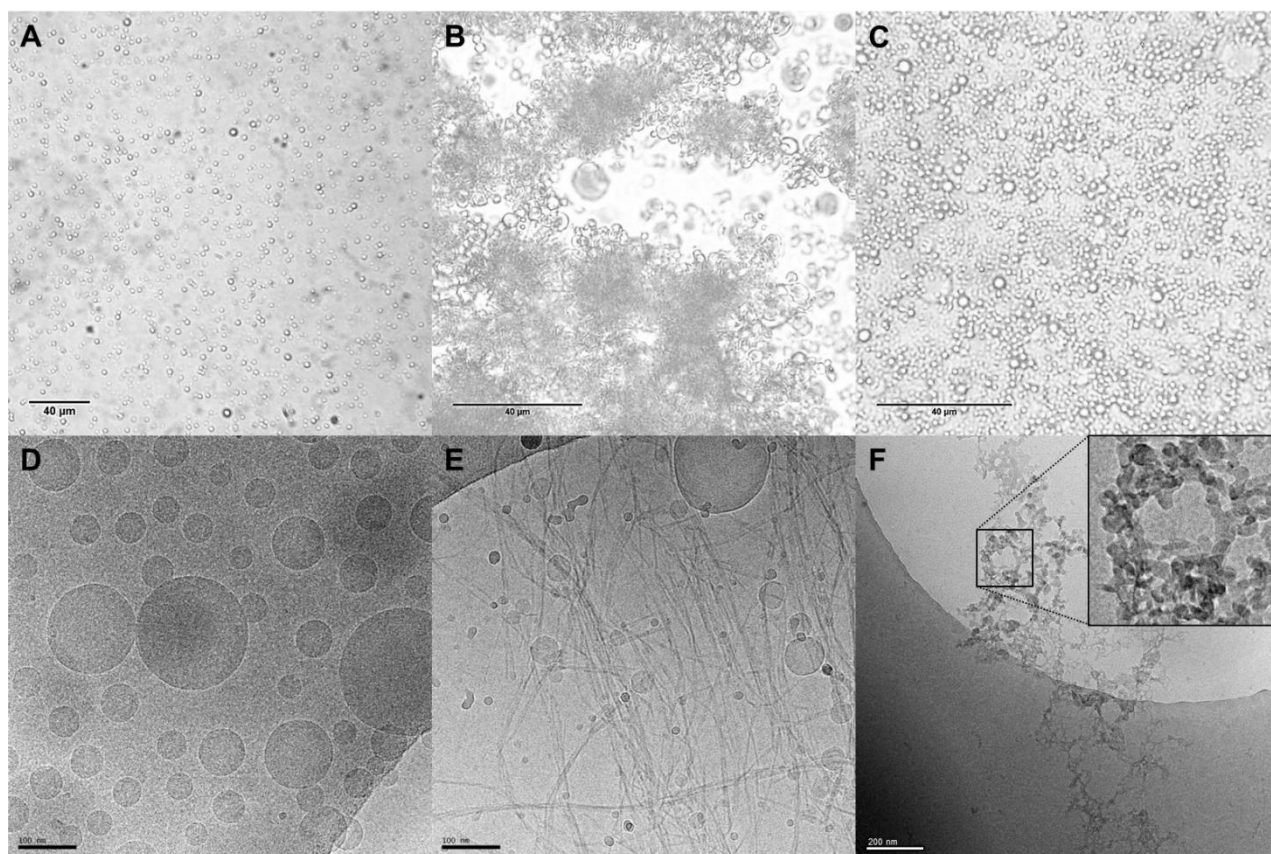
8 <sup>a</sup>Yalkowsky et al., 2003; <sup>b</sup>Mora and Martínez, 2006; <sup>c</sup>Yalkowsky and Dannenfelser, 1992;

9 <sup>d</sup>Lerdkanchanaporn et al., 2001; <sup>e</sup>Levis et al., 2003

10

### 11 3.2.2. Characterization with optical microscopy and cryo-TEM

12 HFBII stabilized emulsion droplets could clearly be observed in optical microscopy as well as cryo-  
 13 TEM images. Optical microscopy images of formulations that were selected for the drug release  
 14 studies were taken after 1:10 dilution as a quality control (Fig. 3 A-C). The formulations contained  
 15 HFBII and combinations of HFBII-NFC-N and HFBII-NFC-O as stabilizers. NFC-O stabilized  
 16 emulsions seemed to be more stable upon dilution in light microscopy images than NFC-N stabilized  
 17 (Fig. 3B-C). NFC-O stabilized emulsions had more uniform distribution of emulsion droplets in  
 18 evenly distributed fiber network whereas NFC-N stabilized emulsions formed clusters of NFC-N and  
 19 emulsion droplets upon dilution. Emulsion droplets with diameters in the micrometer range were  
 20 observed with optical microscopy.



1  
 2 **Figure 3.** Light microscopy and cryo-TEM images of HFBII, HFBII-NFC-N (1:1 mass ratio) and  
 3 HFBII-NFC-O (1:1 mass ratio) stabilized emulsions. Upper image row (A-C) contains light  
 4 microscopy images of respective formulations, whereas lower image row (D-F) contains cryo-TEM  
 5 images. The emulsion droplets of HFBII-NFC-N and HFBII-NFC-O formulations were within the  
 6 entangled NFC fibers. For solely HFBII stabilized emulsions only spherical emulsion droplets were  
 7 observed. Scale bar is 40 µm for A-C, 100 nm for D-E and 200 nm for F image.

8

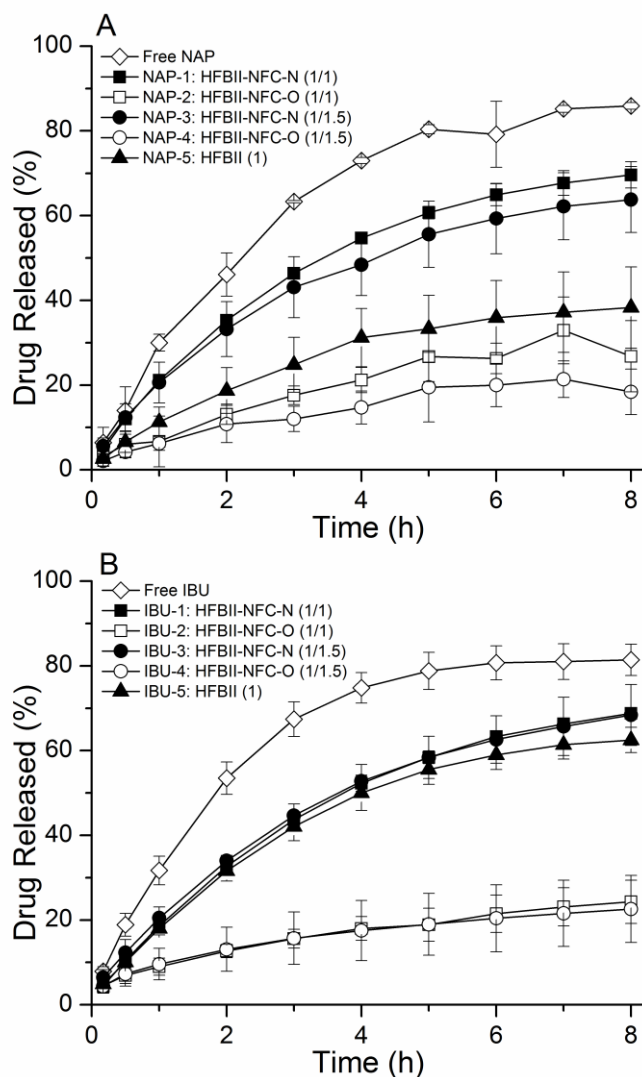
9 The spherical morphology of the protein coated emulsion droplets as well as the wide size distribution  
 10 in all formulations was confirmed with cryo-TEM (Fig. 3D-F). For HFBII-NFC-N formulation the  
 11 fibrous NFC matrix surrounded the emulsion droplets, whereas for solely HFBII stabilized emulsion  
 12 only the protein coated spherical emulsion droplets were observed. The NFC-O fibers surrounding  
 13 the protein coated oil droplets consisted of shorter NFC-O fibers as observed in cryo-TEM images  
 14 (Fig. 3F) as opposed to the longer NFC-N fibers (Fig. 3E). NFC-O fibers are typically shorter than  
 15 NFC-N fibers due to the oxidation process that reduces the fiber length (Benhamou et al., 2014). It  
 16 was observed that the emulsion droplets had a tendency to exist as clusters when stabilized with the  
 17 shorter NFC-O fibers. The expected stabilization mechanism, a combination of HFBII stabilized oil  
 18 droplets with NFC fiber network, of the emulsions was confirmed with cryo-TEM.

19 *3.3. In vitro drug release studies*

1 *In vitro* drug release studies were done in diffusion cells through a 0.4  $\mu\text{m}$  pore size membrane.  
2 Therefore, lag times were observed in the release rate profiles at the beginning of the drug release  
3 studies. The model compounds NAP and IBU both have three distinctive diffusion steps from the o/w  
4 emulsions in our experimental setup that need to be considered when interpreting the drug release  
5 results. These steps include: 1) diffusion across the HFBII coated oil droplet interfacial film, 2)  
6 diffusion through the water phase of the diluted emulsion in the donor compartment and 3) diffusion  
7 from the donor compartment across the membrane to the receptor solution.

8 The drug release profiles of NAP and IBU from solely HFBII stabilized formulations were compared  
9 to NFC containing formulations. The amount and grade of NFC was investigated in terms of effect  
10 to drug release rate. The cumulative drug release of NAP and IBU from the HFBII, HFBII-NFC-N  
11 and HFBII-NFC-O stabilized emulsions are presented in Figure. 3. NAP (pKa 4.15) and IBU (pKa  
12 4.85) are both weak acids and poorly soluble in low pH values but have a high solubility above pH  
13 6.8 due to the ionization (Álvarez et al., 2011;Khadra et al., 2015). Therefore, the diffusion of NAP  
14 and IBU across the HFBII coated oil droplet interfacial films and NFC fiber matrix was evaluated by  
15 using the free drug solutions of NAP and IBU as positive controls for immediate release. The *in vitro*  
16 drug release experiments, conducted under sink conditions, indicated that different formulation  
17 compositions had an impact on the drug release rate when compared to the free drug solution controls  
18 of NAP and IBU. Major differences in the drug release rate were observed depending on the grade of  
19 NFC fibers used in the formulations.

20 NAP release was fast from HFBII-NFC-N formulations and 64-70% was released during 8 h in  
21 comparison to 85% of the free drug through the membrane (Fig. 4). The drug release was slightly  
22 slower than for the positive control of immediate release (free drug solution), but still close to an  
23 immediate drug release profile. Sustained drug release profile for NAP was obtained with the HFBII-  
24 NFC-O formulations as only 18-27% was released during 8 h. The drug release of NAP from HFBII  
25 formulation (NAP-5) resembled a sustained release profile with the 38% of drug release during 8 h.  
26 The amount of NFC-N or NFC-O in the formulation affected only slightly the drug release rates of  
27 NAP. However, the grade of NFC fibers used in the formulations with HFBII had a significant impact.



1

2 **Figure 4.** Drug release studies with NAP and IBU: A) Cumulative NAP release (%) from different  
 3 formulations (mean  $\pm$  S.D., n=3-5), B) Cumulative IBU release from different formulations (mean  $\pm$   
 4 S.D., n=4-5). Mass ratios of HFBII to NFC-N or NFC-O are in brackets.

5

6 The drug release results of NAP and IBU from different formulations were rather similar (Fig. 4).  
 7 IBU release was fast from the HFBII-NFC-N and HFBII formulations. 62-67% of IBU was released  
 8 in 8 h, while 80% of the free drug had passed through the membrane in the same time. Sustained drug  
 9 release profiles were obtained for IBU with HFBII-NFC-O formulations as only 20-23% of IBU was  
 10 released during 8 h. The amount of NFC-N or NFC-O in the formulation did not affect significantly  
 11 the IBU release rates, but the grade of NFC fibers did. Overall, the model drugs were released in a  
 12 sustained manner from the HFBII-NFC-O formulations. By comparison, immediate release profiles  
 13 were gained with the HFBII-NFC-N formulations. It was expected that different release profiles  
 14 would be gained with these materials as it has been previously reported that the drug interactions with

1 HFBII and different NFC-grades has an impact on the drug release rate (Kolakovic et al., 2013;Valo  
2 et al., 2010;Valo et al., 2013). Especially pH dependent electrostatic interactions between drug  
3 molecules and NFC fibers seem to play an important role with regard to drug release (Kolakovic et  
4 al., 2013). It is possible that some drug interactions existed with both NFC grades driven by aromatic  
5  $\pi$  stacking and hydrogen bonding. However, physical interactions such as viscosity, stability and the  
6 surface barrier of the emulsion droplets were most likely more critical in determining the drug release  
7 rates.

8 At pH 7.4 both NFC grades are negatively charged, but NFC-O has a greater negative charge due to  
9 a greater distribution of carboxyl groups directly at the fiber surface (Saito et al., 2007;Weishaupt et  
10 al., 2015). HFBII has an isoelectric point (pI) of 6.7 and a negative net charge at higher pH values  
11 (Krivosheeva et al., 2013). However, it is possible that the protein coated oil droplets may have had  
12 a positive charge at pH 7.4 as the formation of tight interfacial HFBII film reportedly reduces the  
13 surface area and ionizable functional groups exposed to water resulting in a shift of the pI (Basheva  
14 et al., 2011). The measured zeta potential values of HFBII stabilized preliminary formulations  
15 indicated positive net charge at neutral environment of ultrapure water (Table 2). It is likely that the  
16 sustained release profiles of NAP and IBU from HFBII-NFC-O formulations were attributed to a  
17 greater diffusion barrier at the o/w-interphase that was present due to electrostatic attraction between  
18 negatively charged NFC-O and positively charged HFBII. As NFC-N has a lower negative charge,  
19 the effect was not as pronounced resulting in an immediate drug release profiles.

20 Furthermore, NFC-O stabilized emulsions were more stable upon dilution (Fig. 3B-C, 10x dilution)  
21 and the emulsion structure remained more intact than with NFC-N. This was probably attributed the  
22 high surface density of negative charges of NFC-O fibers due to multiple carboxyl groups on the fiber  
23 surface (Benhamou et al., 2014). Since the emulsions were slightly diluted (4x) in the release  
24 experiments, the electrostatic repulsion between NFC-O fibers may have stabilized the emulsion  
25 droplets of HFBII-NFC-O formulations more effectively in addition to electrostatic attractive  
26 interactions between HFBII and NFC-O. Furthermore, NFC-O had a higher viscosity than NFC-N  
27 (Fig.2) indicating that NFC-O was a more effective grade for emulsion storage stabilization due to  
28 increased viscosity of the continuous phase. In theory, the higher viscosity of HFBII-NFC-O  
29 formulations may have prolonged the drug release, but upon dilution the effect should have been  
30 minimal. We believe that the sustained drug release properties of HFBII-NFC-O formulations were  
31 at least partially attributed to the electrostatic interactions between HFBII and NFC-O, which  
32 stabilized the emulsion structure.

1 For solely HFBII protein stabilized formulations an immediate and sustained drug release profiles  
2 were observed for IBU and NAP respectively. IBU has a higher solubility at pH 7.4 than NAP (see  
3 Table 4.). The solubility difference at pH 7.4 may be the reason for the differences in the drug release  
4 rates of these molecules. The solubility of the diffusing drug molecules can reportedly have a higher  
5 effect on drug release rate than the interfacial film properties of the emulsion droplets (Llinàs et al.,  
6 2013). Consequently, the solubility of the ionized drug molecules, in the pH 7.4 release medium, can  
7 affect the diffusion rate from the inner oil phase and overall drug release. Therefore, it was concluded  
8 that while the HFBII formulations were successful in controlling the drug release to some extent it  
9 was beneficial to add NFC into the formulations to gain well defined release profiles. Furthermore,  
10 the storage stability of the emulsions was improved when NFC was used in the stabilization together  
11 with HFBII (Fig. 1).

12 HFB proteins have hydrophobic and hydrophilic patches directly at their surfaces (Lumsdon et al.,  
13 2005;Paananen et al., 2013;Szilvay et al., 2007). The amphiphilic nature of HFBII has been exploited  
14 for hydrophilic and hydrophobic interactions with interfaces. Previously the ability of HFBs to  
15 modify hydrophobic interfaces into hydrophilic ones has been used in nanoencapsulation of  
16 hydrophobic nutraceuticals, stabilization of emulsions and hydrophobic drug nanoparticles (Israeli-  
17 Lev and Livney, 2014;Valo et al., 2010;Varjonen et al., 2011). Here HFBII was mainly used to  
18 stabilize the o/w interphase of pharmaceutical emulsions and to aid in the solubilization of BCS class  
19 II drugs in the oil phase of emulsions. Some hydrophobic and hydrophilic interactions between HFBII  
20 and the model drugs may have existed, but the effect on drug release profiles was minimal. Similar  
21 release profiles were observed from the HFBII and HFBII-NFC-N formulations apart from NAP with  
22 HFBII. The release profiles of IBU and NAP were close to an immediate release profiles from HFBII-  
23 NFC-N. At the low concentrations of 1 mg/ml for HFBII and 1 - 1.5 mg/ml for NFC-N in the  
24 formulations, these materials were better suited for emulsion storage stabilization than to provide  
25 controlled release. However, immediate release profile after oral administration is beneficial in many  
26 pharmaceutical applications such as analgesia.

27 Based on the *in vitro* drug release studies, it was clearly shown that the use of native or oxidized NFC  
28 as an emulsion stabilizer results in different drug release profiles. Morphology and the stability of the  
29 different formulations most likely had an impact on drug release results. Drug release profiles were  
30 affected only moderately by the viscosity of the emulsions or by the interactions of the drug molecules  
31 with NFC fibers and HFBII proteins. Clearly both NFC grades, native and TEMPO oxidized, can be  
32 used as emulsion stabilizers. However, further studies are needed in order to evaluate a concentration  
33 range where HFBII may act as a surfactant and provide sustained release from the inner oil phase.

1 The oxidized NFC grade seems promising excipient for sustained release formulations as an emulsion  
2 stabilizer due to its greater extent to modify the drug release rate. The native NFC grade in turn seems  
3 more suitable for formulations that benefit from an immediate drug release profile. As an increasing  
4 number of drug candidates suffer from poor aqueous solubility, the emulsion formulation is an option  
5 for solubilization of lipophilic drugs and enhancement of the adequate absorption from the GIT  
6 following oral administration.

#### 7 **4. Conclusions**

8 This study has shown that a combination of natural biopolymers HFBII and nanofibrillated cellulose  
9 can be used to tailor emulsions for immediate and sustained drug release applications. The low  
10 toxicity of these materials is a significant benefit in drug delivery applications via the oral route when  
11 compared to synthetic traditional surfactants. The formulations that contained HFBII and oxidized  
12 nanofibrillated cellulose proved to be effective in controlling the drug release rate, which indicated  
13 the suitability of these materials for controlled drug release applications. Formulations with HFBII  
14 and native nanofibrillated cellulose were more suitable for immediate drug release applications. The  
15 drug release profiles of our poorly soluble model compounds, naproxen and ibuprofen, could be tuned  
16 by the choice of the nanofibrillated cellulose grade.

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24

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