FABRICATION AND CHARACTERIZATION OF A MAGNETIC BACTERIAL NANOCELLULOSE FOR NEUROVASCULAR RECONSTRUCTION OF CEREBRAL ANEURYSMS

BY

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THESIS

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ABSTRACT

A cerebral aneurysm is a condition where a defect protrudes out the arterial wall, and which is formed due among other reasons to abnormal high hemodynamic stresses that contribute to deterioration and dilation of the blood vessel. A desirable treatment of cerebral aneurysms is the complete cut-off of the defect from the parent artery with minimum luminal obstruction. Even though different approaches have shown to exclude the defect from the parent artery, the main shortcoming remains the delayed reconstructive occlusion of the aneurysm, which occurs a period of weeks to months. We hypothesized that a material with magnetic properties can provide the force required to speed up re-endothelization across the aneurysm defect, since it can facilitate high cell density coverage at the damaged site in virtue of its ability to capture and retain magnetically functionalized endothelial cells. The aneurysmal neck is a hostile environment for tissue growth resulting from the blood’s shear stress that precludes cell adhesion and proliferation. Therefore, this strategy looks for designing a magnetic material for rapid endothelial cell take up and retention against hemodynamic forces. This magnetic material is required also to satisfy other important features such as biocompatibility, appropriate mechanical properties (e.g. tensile strength and compliance), and blood compatibility (non-thrombogenic).

In the present work, we have used bacterial nanocellulose (BNC) as starting material for the production of a magnetic hydrogel, which we named magnetic bacterial nanocellulose (MBNC). BNC is a natural polymer produced by the bacterial strain *Acetobacter xylinum*, which is extruded as a pellicle in the interface liquid/air to protect the bacteria from dehydration and UV radiation. BNC possesses a multiple of desirable physical and chemical properties for tissue engineering applications such as biocompatibility, high swell ratio, and high tensile strength. D-glucose chains abundant in hydroxyl groups conform the BNC’s chemical structure, which are able to adsorb metallic ions and compounds with functional groups active on hydrogen-bonding formation. A brief review about the BNC and magnetic hydrogels are presented in chapter I.

In chapter II, we describe the production of BNC and its purification to subsequently synthesize the MBNC through an *in-situ* precipitation method, in which superparamagnetic iron oxide nanoparticles (SPION) are formed inside the BNC by using ammonium hydroxide as
precipitating agent. Different concentrations of Fe$^{3+}$ and Fe$^{2+}$ iron salts were used for the synthesis of MBNC, and their effect on BNC permeability, porosity and magnetic saturation were analyzed. The permeability testing was performed using a side-by-side diffusion cell. MBNC porosity was estimated using a mass equation balance. Magnetization testing was performed using vibrating sample magnetometer. Scanning electron microscopy (SEM) and magnetic force microscopy (MFM) were used to reveal the morphology and magnetic domains on MBNC respectively. Chemical characterization of the MBNC was performed via X-ray photoelectron spectroscopy (XPS). Because naked SPION are easily oxidized to Fe$_2$O$_3$ under environmental conditions, dextran was used to coat the SPION embedded into the MBNC.

In chapter III, once established the optimal reaction conditions for the MBNC synthesis, MBNC pellicles were tested for biocompatibility and cell capture under dynamic fluid flow conditions. Cell adhesion sites were introduced on the surface of MBNC via collagen-conjugation using CDAP as activating agent. Our results showed a satisfactory MBNC magnetization, which was able to separate magnetically functionalized cells under dynamic flow conditions compare to non-magnetized MBNC.
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1.1 Background and motivation

The treatment of intracranial aneurysms requires a rapid and permanent exclusion of the defect from the parent artery perfusion to prevent its rupture in the short term and avoid its recurrence or recanalization in a long term. A randomized study conducted by the International Subarachnoid Aneurysm Trial (Molyneux et al. 2002) validated endovascular treatment using coiling procedures as an acceptable treatment modality for patients with intracranial aneurysms, in terms of a significant survival up to 1 year after clinical intervention, as compared to those patients that underwent the alternative of surgical clipping.

Despite endovascular coil occlusion is associated with suitable results preventing hemorrhage during the acute phase of subarachnoid hemorrhage (Hayakawa et al, 2000), residual filling and aneurysmal neck remnants are the major concerns of this approach (Raymond et al, 2003; Kole et al, 2005; Gorji et al, 2001). Different strategies such as stent grafts have emerged in an effort to overcome the suboptimal aneurysmal sac occlusions and post-coiling recurrences. The main advantages of these approaches can be summarized as (i) reduction of the risk associated with an aneurysm packing with coils; (ii) the immediate flow disruption of both inflow and outflow inside of the aneurysmal defect; iii) its inherent thrombogenicity; and (iv) time and cost advantages (Nishi et al. 2003; Fiorella et al. 2009).

However, many of the current approaches failed to confine the thrombogenic activity extraluminally in the aneurysmal sac and reestablish a desirable cut-off of the defect from the parent artery (Allain et al. 2013). Uncovered stents formed by porous metallic strands such as pipeline embolization device (PED) has provided a stand-alone stent able to terminate the aneurysm by progressively disrupting the flow inside the defect, and by encouraging endovascular reconstruction across the aneurysmal neck defect (de Barros Faria et al. 2011). Clinical trials in selected intracranial aneurysms in humans using PED showed a complete angiographic exclusion of the defect up to 93% of cases by the 6 months follow-up evaluation, supporting endothelialization of the neck defect.
(Lylyk et al. 2009). Despite this accomplishment in intracranial aneurysmal treatment, the main shortcoming of PED remains the delayed reconstructive occlusion of the aneurysm, which occurs in a period of weeks to months.

A rapid and curative reconstruction of the absent tunica media in the aneurysmal neck requires an optimal biological environment in favor of facilitating neointimal overgrowth across of the defect, and thus, a complete and durable obliteration of the aneurysm. Since neck defect is a hostile environment for tissue regeneration resulting from the blood’s shear stress, the recruitment of a cell nidus through a focal magnetic field could provide the force required for initial cell adhesion and proliferation, and final endothelialization of the absent tunica media.

The formation of a cell nidus across the aneurysm neck can be achieved by using a stent-coating material with suitable biological and mechanical properties including biocompatibility, high tensile strength, compliance, and blood compatibility (Fink et al. 2011). Synthetic polymers such as poly(ethylene terephthalate) (Dacron) and poly(tetrafluoro ethylene) (Teflon) have been successfully used for the replacement of large-diameter arteries (> 6 mm of internal diameter); however, these polymers fail to replace small-diameter arteries since they contribute to the formation of thrombosis and intimal fibrous hyperplasia. The low performance of Dacron and Teflon in treating injuries on small diameter arteries has been attributed primarily to the compliance mismatch between these polymers and the native arteries, which contributes to altered hemodynamic loads at the anastomotic site (Seifu et al. 2013; Fink et al. 2012).

Current strategies for the treatment of injuries in small diameter arteries look for creating scaffolding materials able to mimic the native architecture of the blood vessels, which may have added functionalities to enhance the device performance. Those include, for example, biodegradable polymers as poly(caprolactone) bearing either growth factors, antibodies, peptides, or magnetic molecules to provide better homing and patency of the scaffolding material (Melchiorri et al. 2013). Among the natural polymers, bacterial nanocellulose (BNC) has gained considerable attention because of its mechanical and morphological properties mimic those of the native arteries (Fink et al. 2011). The nanofibril architecture of the BNC revealed via scanning electron microscopy (SEM) shows a size and structural organization that resembles that of the type I collagen (Fink et al. 2011).
Because vascular devices such as stent and vascular grafts may take several months to achieve a re-endothelialization at the injured site (Pislaru et al. 2006), cell-based therapies have been used not only to expedite re-endothelialization but also to achieve an active antithrombotic surface able to protect the vascular devices from platelet activation, reducing the risk of hyper-thrombotic formation. For example endothelial cells, which form the inner layer of the blood vessels, facilitate the transit of plasma and preserve the blood fluidity by different anticoagulant and antiplatelet mechanisms (Rajendran et al. 2013). It has been shown that delivery of endothelial progenitor cells reduces restenosis and shortens the time required for achieving satisfactory re-endothelialization in vascular grafts, showing even higher performance compare to drug-eluting stents (Douglas et al. 2013). Vascular smooth muscle cells (VSMC) have also been used to promote the formation of neointima in experimental aneurysms in dogs (Raymond et al. 1999). VSMC are the main collagen-synthetic cells in the arteries, and they play a pivotal role in vessel tone. Additionally, VSMC may limit the arterial wall remodeling by triggering endothelial cell repopulation and endoluminal healing, which are conditions required for a complete exclusion of the aneurysm defect, and for preventing complications related to the thrombus spreading (Gomes et al. 2001; Allaire et al. 2004). Despite those features, VSMC deregulation and overgrowth can lead to intimal hyperplasia restraining its clinical use (Melchiorri et al. 2013).

1.2 Hypothesis and objectives

Limited effort has focused on promoting a rapid and localized endothelialization at the cerebral aneurysmal neck orifice rather than promoting defect thrombogenicity, although there have been some promising results reported using cell-based therapies combined with material functionalization strategies especially in experimental coronary artery injuries, whose pathology is close similar to that observed in aneurysmal defects (Melchiorri et al. 2013). Allain and colleagues briefly outlined the development of a neuroendovascular treatment alternative that required five strategic elements: “1) use of the stent’s inherent thrombogenicity, 2) use of stents as scaffolds, 3) the need of tissue nidus to repair a vascular defect across a scaffold, 4) using the arterial wall as a source of stem cell derivatives for tunica media reconstruction, and 5) confining induced thrombogenic activity extraluminally and the role of a focal magnetic field.” It was based on these strategic elements that Allain and colleagues conjectured the emergent role of nanotechnology in developing this neuroendovascular strategy introducing a nano-to-micro scale approach involving bioactive
functionalized coatings that would promote cellular adhesion, migration, and proliferation along vascular defects (Allain et al. 2013). Taking into account those five strategic elements, we hypothesized that a focal magnetic configuration could enable a controlled and efficient immobilization of magnetically functionalized cells with the necessary mechanical support, high initial cell density, and proper cell distribution to promote cell adhesion and tissue growth across the aneurysm neck. An efficient magnetic cell functionalization and targeting process might maximize the available magnetic force, and maintain cell functionality necessary for cell retention and attachment based on the strategy described by Allain and colleagues.

The goals of this work are thus to support the arterial wall reconstruction across the aneurysmal neck using a magnetic scaffold based on BNC to effectively target cells to the aneurysmal defect under pulsatile flow conditions. The magnetic BNC (MBNC) is achieved by in situ precipitation of Fe$^{3+}$ and Fe$^{2+}$ into the BNC, which is used as a template for nucleation and growth of superparamagnetic iron oxide nanoparticles (SPION), so that a three-dimensional magnetic hydrogel is formed. Cells are magnetically functionalized with SPION via passive uptake. The feasibility of this approach is tested under pulsatile flow condition by using a parallel-plate flow chamber operating in the range of physiological shear stresses.

1.3 Approach

In this study, we have used in situ precipitation of Fe$^{3+}$ and Fe$^{2+}$ into the BNC to render this natural hydrogel magnetic. Because the BNC is rich in hydroxyl groups, those allow the adsorption of metallic ions contributing to the nucleation, growth, and dispersion of SPION along the BNC nanofibril network. Dextran coating was used on the MBNC and its performance regarding particle size and dispersion was analyzed via scanning electron microscopy (SEM) and vibrating sample magnetometer (VSM). Dextran possesses anticoagulant properties, which makes it suitable for blood-contact applications. Magnetic regions on synthesized MBNC were revealed via magnetic force microscope (MFM). The permeation coefficient of MBNC pellicles was calculated using the Fick’s second law and a horizontal diffusion chamber.

We have also performed studies of biocompatibility and cellular capture on MBNC using parallel-plate flow chamber. Because natural BNC lacks cell adhesion sites, we activated the hydroxyl
groups exposed on the surface of the BNC and MBNC via 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) to covalently bind collagen to the BNC and MBNC fibrils.

1.4 References


CHAPTER 2
SYNTHESIS AND CHARACTERIZATION OF THE MAGNETIC BACTERIAL
NANOCELLULOSE

2.1 Introduction

Bacterial nanocellulose (BNC) is a natural polymer synthesized by the bacteria Acetobacter xylinum, which forms a pellicle at the interface liquid/air, and it is believed to provide UV radiation and desiccation protection to the bacterial colonies (Römling & Galperin 2015). The chemical structure of the BNC is equivalent to that of the plant cellulose, i.e. D-glucose molecules, but a three-dimensional network with fibrils in the nanometer range characterizes the former (Fink et al. 2012; Hu et al. 2014). Additionally, BNC is free of impurities such as biogenic compounds, e.g. lignin, pectin, and arabinan; which are found in plant cellulose and other cellulose sources (Fink et al. 2012). BNC possesses other attractive physical and chemical properties such as high mechanical strength and a high degree of polymerization. The Young’s Modulus of a single filament of bacterial cellulose has been estimated around 114 GPa (Hsieh et al. 2008). The degree of polymerization of BNC varies from 300 to 10,000, which depends on mainly the culturing conditions, the presence of additives in the culture medium, and bacterial strain (Campano et al. 2015).

BNC pellicles adopt the length and width of the growth culture vessel, and their supramolecular structure such as porosity, fibril alignment, and mechanical properties can be modified either during the fermentation process (biosynthetic) or be subject to a post-treatment method via chemical and physical means. The biosynthetic modification consists mainly in introducing additives to the culture medium during the incubation period, which do not incorporate in the final chemical composition of the cellulose. For example, silicone oil has been used to cover the upside face of the culture medium in order to get a higher degree of fibril orientation and swelling capacity (Putra et al. 2009). Other types of additives such as carboxymethylcellulose, poly(vinyl alcohol) and poly(ethylene glycol) have been used to tune the porosity and water retention capabilities of the BNC (Hu et al. 2014)
Different mechanisms for surface chemical modification and preparation of BNC-based nanocomposites have also been investigated in order to add different functionalities and extend the number of applications of the BNC (Hu et al. 2014). The BNC can be carboxymethylated, acetylated and phosphorylated through means similar to those used in plant cellulose. Acetylation has been employed to provide enhanced adhesion properties of the BNC to hydrophobic matrices (Kim et al. 2002). Conjugation of proteins to the BNC backbone has been implemented using activating agents such as 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) (Kuzmenko et al. 2013).

Preparation of BNC-based nanocomposites can be achieved through in-situ and ex-situ synthetic means. Both approaches take advantage of the extensive surface area provide by the BNC nanofibril structure and its abundance in hydroxyl groups, which function as a soft template for the adsorption of metal ions and polymeric compounds via proton exchange reactions (Hu et al. 2015; Olsson et al. 2010). Hydroxyl groups possess a very strong affinity and medium affinity to ferric and ferrous ions respectively (Qiao et al. 2009). Early studies performed by Belford (1958) showed that cations appear to be exchanged for hydrogen regardless of the cellulose’s source and the type of metallic cation, which was demonstrated by detecting changes in the pH of the solution containing both the cellulose and the metallic cation.

In-situ methods allow the nucleation and growth of nanoparticles using the BNC as a dispersing agent, whereas ex-situ methods use nanoparticles that are already formed to impregnate the BNC. Magnetically actuated hydrogels have been prepared by in-situ methods by precipitating cobalt ferrite nanoparticles on BNC (Olsson et al. 2010). Those magnetic hydrogels can be used to remotely release drugs on demand, as well as take up cells, growth factors and proteins functionalized with magnetic nanoparticles (Li et al. 2013). Ex-situ methods have been employed to impregnate the BNC with silica nanoparticles for applications in drug/gene delivery and sensing devices (Ashori et al., 2012).

In the present work, we have used BNC as a template for the in situ precipitation of superparamagnetic iron oxide nanoparticles (SPION) (magnetite, \( \text{Fe}_3\text{O}_4 \)) to form a magnetic hydrogel, which we have named magnetic bacterial nanocellulose (MBNC). The MBNC is envisioned to act as cell nidus for initial cell retention at the site of the aneurysmal neck defect,
which is a hostile environment for re-endothelization due to the high hemodynamic stresses at injured site. Additionally, MBNC may be used as a magnetically actuated platform for drug delivery.

During the MBNC synthesis, an aqueous solution containing decreasing concentrations of Fe\textsuperscript{2+} and Fe\textsuperscript{3+} ions were investigated in terms of SPION dispersion within the BNC pellicle, nanoparticle size, and MBNC magnetic saturation. Dextran was used to coat the SPION embedded on the MBNC. Characterization of the resulting magnetic hydrogel was performed via scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), vibrating sample magnetometer (VSM), and magnetic force microscopy (MFM). Finally, the permeability of the BNC and MBNC was studied via a horizontal diffusion chamber using lysozyme and bovine serum albumin as marker molecules. The permeability properties of the MBNC are important for two main reasons: (1) to determine the diffusion rate of blood components towards or out of the aneurysmal sac, and (2) to gain knowledge about the transport mechanisms of nutrients and waste products require for cell encapsulation and drug delivery systems.

2.2 Results and Discussion

2.2.1 MBNC synthesis, morphology and chemical composition

SPION are highly hydrophobic in nature, possess a high surface energy, and are unstable under ambient conditions, therefore, tend to form aggregates and oxide towards Fe\textsubscript{2}O\textsubscript{3} reducing its superparamagnetic properties (Gupta & Gupta, 2005). The addition of surfactants such as dextran not only can help to control the size and colloidal stability of SPION in aqueous solutions, but also improve their biocompatibility and circulation time in blood (Laurent et al. 2008). Dextran is a polymer of anhydroglucose, which is water soluble and biocompatible and possesses antithrombotic (anti-platelet) properties. Dextran (DXT) strongly sticks to the SPION surface via electrostatic attraction through its hydroxyl groups. DXT can prevent aggregation of SPION due to the steric and electrostatic repulsive forces between capped nanoparticles (Laurent et al. 2008; Hajdu et al. 2008). Fig. 2.1 (a) and (b) show the chemical structure of the DXT and the BNC respectively.

To synthesize the MBNC, one BNC pellicle was immersed in a solution containing Fe\textsuperscript{3+} and Fe\textsuperscript{2+}, and let adsorb these cations for 15 min before nanoparticle formation (Fig. 2.1 (c)). SPION
were then formed along the BNC fibrils by adding ammonium hydroxide to the reaction. Decreasing molar concentrations of iron salts (100 mM, 50 mM, and 25 mM) were prepared to investigate the optimal SPION concentration in terms of dispersion and magnetic properties. Once the SPION formed, the MBNC was placed in a dextran solution. Incubation times for DXT were adjusted so that this polymer could penetrate the BNC pellicle deep enough to coat the inner SPION, and not limiting the coating to the outermost nanoparticles in the MBNC. Those times were determined by considering the typical transport rates of marker molecules of similar molecular weight such as lysozyme (14,307 kDa) on unmodified BNC (Sokolnicki et al. 2006).

**Figure 2.1.** Chemical structure of (a) dextran (DXT) and (b) bacterial nanocellulose (BNC); (c) the hydroxyl groups on BNC allows for the adsorption of metals ions (Fe$^{3+}$ and Fe$^{2+}$) and functions as a template for the MBNC synthesis.
Fig. 2.2 shows the morphology for the MBNC at decreasing molar concentration of iron salts and capped with dextran, along with unmodified BNC (Fig. 2.2 (a)). The native structure of the BNC is characterized by a sojourn of fibers with an average diameter of 30 nm, without well-defined pore structures. Fig. 2.2 reveals that SPION formed along the BNC fibrils as expected for the BNC acting as a template for nanoparticle nucleation and growth (Hu et al, 2015). The higher concentration (100 mM) yielded the highest densely packed SPION (Fig. 2.2 (d)). This same tendency has been observed using FeSO₄/CoCl₂ as precursors molecules in the formation of cobalt ferrite nanoparticles embedded on BNC (Olsson et al. 2010). On the other hand, concentrations of 50 mM and 25 mM showed high-spaced SPION or uncoated areas in the BNC fibrils (Fig. 2.2 (b) and (c)). The nanoparticle size in the different MBNC preparations using DXT as capped polymer is about 30 nm.

Figure 2.2. Scanning electron micrographs of (a) BNC, (b) 25 mM MBNC, (c) 50 mM MBNC, and (d) 100 mM MBNC
The chemical composition of the MBNC revealed via XPS is shown in Fig. 2.3. This figure reveals that the binding energy of Fe 2p$_{3/2}$, which corresponds to Fe$^{2+}$ and is about 709 eV, switched to 711 eV indicating the formation of Fe$_3$O$_4$ or magnetite in the BNC (Wagner et al. 1979).

![Chemical composition of the MBNC revealed via XPS. A binding energy of Fe 2p$_{3/2}$ at 711 eV indicates an increase in the oxidation state of Fe$^{2+}$.](image)

**Figure 2.3.** Chemical composition of the MBNC revealed via XPS. A binding energy of Fe 2p$_{3/2}$ at 711 eV indicates an increase in the oxidation state of Fe$^{2+}$.

### 2.2.2 Swelling ratio, porosity and permeability

The swelling ratio for unmodified BNC was estimated to be 66.08 ± 15.35 with a porosity of 98 %, this latter value being in agreement with the that reported by other researchers (Sokonicki et al. 2006). The high porosity registered by the BNC indicates that the transport of solutes across the membrane may take place majorly through the fluid-filled space. Indeed, by measuring the diffusivity of dextran at various molecular weights, Sokonicki and coworkers (2006) showed that the pore mechanism is the major contributor for solute transport across the BNC, thus, a reduction in diffusivities can be attributed to hydrodynamic and exclusion means due to collisions with the BNC fibers.
Knowledge about the transport mechanisms on MBNC is important for determining the barrier properties of the membrane so that the blood flow in and out of the aneurysm neck can be completely disrupted without compromising the transport of nutrients necessary for cell viability. On the other hand, magnetic control of the MBNC porosity can be used for on-demand drug-delivery such as the controlled release of angiogenesis factors.

Given that the incorporation of SPION on the BNC network reduces the void space available for diffusion, we expect a reduction in the permeability coefficients for solutes with increasing molecular weight. As an alternative, magnetic control of the MBNC porosity might open or close the BNC structure to control the passage of solutes.

Table 2.1 summarizes the results for the calculated permeability coefficients on MBNC with decreasing iron salt concentration for marker molecules with different molecular weights. To estimate the permeation coefficients, we used the Fick’s second law assuming a pseudo-steady state. The measurements were performed using a side-by-side diffusion chamber where both reservoirs were continuously stirred using a magnetic bar to avoid protein sedimentation. Since the solutions were exposed to a permanent magnetic field, in this case, the hot plate used to stir the solutions, we envisage under these conditions, a pore-close structure for the MBNC.

Table 2.1. Permeation coefficients (P) calculated for bovine serum albumin (BSA) and Lysozyme (LZ) as marker molecules across MBNC pellicles with SPION concentrations of 100 mM, 50 mM and 25 mM.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Permeation Coefficient</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P_{\text{BSA}}$ [x 10^{-7} \text{cm}^2/\text{s}]</td>
<td>$P_{\text{LZ}}$ [x 10^{-7} \text{cm}^2/\text{s}]</td>
</tr>
<tr>
<td>MBNC_100mM</td>
<td>0.734</td>
<td>3.089</td>
</tr>
<tr>
<td>MBNC_50mM</td>
<td>0.697</td>
<td>3.99</td>
</tr>
<tr>
<td>MBNC_25mM</td>
<td>1.278</td>
<td>2.59</td>
</tr>
</tbody>
</table>

The concentration profile for the data presented in Table 1 is illustrated in Fig. 2.4-2.5, for two marker proteins: lysozyme (LZ) and bovine serum albumin (BSA). Notice from Table 1, that even though we were expecting a linear reduction on the permeation coefficients for high molecular weight and high SPION concentrations, such relation is not clear at least for the diffusion of
lysozyme, where the MBNC with the lowest SPION concentration reports the lowest permeation coefficient. This result may indicate that the SPION on the MBNC are not homogeneously distributed, showing regions of low and high-density SPION concentrations, which can facilitate or reduce the diffusion of molecules respectively.

Figure 2.4. Concentration profile of BSA in the receptor reservoir in a side-by-side diffusion cell. The BSA diffuses across the MBNC pellicle to reach the receptor reservoir, which is at lower concentration compare to the donor reservoir.
Figure 2.5. Concentration profile for LZ in the receptor reservoir of a side-by-side diffusion cell.

In Fig. 2.4-2.5 is noted that exists an induction period where the concentration of protein in the receptor reservoir remains invariable or relatively low during the first 20 hours. This delay represents the time for the protein to break through the MBNC mesh-like structure and is observed to be dependent on the protein molecular weight as expected for a mass transport based on pore mechanism.
2.2.3 Magnetic properties

The curve of magnetization versus magnetic field strength for 100 mM MBNC is shown in Fig. 2.6. Notice that no hysteresis loops formed, meaning that at room temperature and in an alternating magnetic field, the MBNC exhibits a superparamagnetic behavior (Filipcesei et al. 2007). This behavior can be described by the Langevin function, which predicts that the magnetization of a superparamagnetic material is directly proportional to the concentration of magnetic particles regardless if they are rigidly fixed to the material as in the case of the MBNC (Filipcesei et al. 2007).

![Graph of Magnetization vs. Magnetic Field](image)

**Figure 2.6.** Magnetization vs. magnetic field strength for 100 mM MBNC. The MBNC shows superparamagnetic behavior and has a maximum saturation magnetization about 10 emu g⁻¹.

High magnetic saturation is desirable for ferromagnetic and superparamagnetic materials that are used for magnetic separation of biocomponents such as SPION-loaded cells since they can provide
high flux gradients up to the maximum material saturation by increasing the magnetic strength of an external uniform magnetic field (Han & Frazier, 2004). Poor magnetic saturation, on the other hand, does not provide the means to increase the magnetic force on magnetically functionalized biocomponents.

Fig. 2.7 shows the magnetic domains in MBNC, with regions of high and low magnetization density in yellow and green color respectively. This image was captured for a bare MBNC (non-DXT coated).

Figure 2.7. Magnetic domains on MBNC visualized via magnetic force microscope. High and low density of magnetic field are observed as yellow and green regions respectively.

2.3 Concluding remarks

We have fabricated a magnetic bacterial nanocellulose or MBNC with a magnetic saturation about 10 emu g$^{-1}$, which is above the value considered to be relevant to biomedical applications. We compared different concentrations of iron salts impregnated on the BNC in terms of nanoparticle size and distribution. We found that using a concentration of 100 mM of Fe$^{3+}$ yielded a fully SPION-covered MBNC. The SPION aligned along the BNC fibers, as expected for the BNC acting as a template for iron salt absorption, nucleation, and growth.
2.4  Materials and methods

2.4.1  Synthesis and purification of the BNC

Note: All the reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Liquid culture medium was prepared by adding 25 g of yeast extract, 15 g of peptone, and 125.0 g of mannitol to 500 ml of high purity water. 2 ml per well of liquid medium was then transferred to a 24-well culture plate and 2 colonies of A. xylinum (ATCC, USA) were inoculated into each well. The 24-well culture plate was then incubated at 30°C for 7 days. At the end of the incubation period, BNC pellicles were extracted and incorporated in a solution of 1% sodium hydroxide for 1 h at 50°C. BNC pellicles were subsequently collected, washed using high purity water and autoclaved 121°C for 20 min (Arias et al, 2016).

2.4.2  Preparation of dextran coated MBNC

BNC pellicles with 2 mm in diameter where immersed in a solution at 3 different concentrations of iron salts (iron (III) chloride hexahydrate and iron (II) chloride tetrahydrate) maintaining a molar ratio $\text{Fe}^{3+}/\text{Fe}^{2+}$ equal to 2 (Table 2.2). The total volume of the iron salt solution was held on 20 ml, which was prepared using deoxygenated high purity water, i.e. milli-Q water bubble with nitrogen gas. The iron salts were allowed to soak into the BNC for 15 min at 70°C silicone oil bath. The synthesis was performed under nitrogen atmosphere and stirred with a magnetic bar to 700-rpm. Depending on the iron salt concentration, either 5 mL or 2.5 mL of $\text{NH}_4\text{OH}$ was added by dropping using a syringe. Immediately after, 2 ml of a dextran solution (MW 9-1.1 kDa) at a concentration of 0.3 g/ml was added to the vessel. The reaction was left to proceed for 30 min. The MBNC and IONP were washed several times with water until a neutral pH was achieved. A detailed description of this protocol is found in the work of Arias and colleagues (2016). The experimental setup is shown in Fig. 2.8.
Table 2.2. Preparation of MBNC using 3 decreasing concentration of iron salts, while conserving a molar ratio equal to 2 and a total volume of 20 ml.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe$^{3+}$ (mg)</th>
<th>Fe$^{2+}$ (mg)</th>
<th>Fe$^{3+}$ [mM]</th>
<th>Fe$^{2+}$ [mM]</th>
<th>Dextran Solution (ml)</th>
<th>Ammonium hydroxide (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dxt_0.1M</td>
<td>541</td>
<td>199</td>
<td>100</td>
<td>50</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Dxt_0.05M</td>
<td>270</td>
<td>99.4</td>
<td>50</td>
<td>25</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>Dxt_0.025M</td>
<td>135.2</td>
<td>49.7</td>
<td>25</td>
<td>12.5</td>
<td>2</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The macroscopic appearance of the BNC before and after loading with Fe3O4 is illustrated in Fig. 2.8 (b) and (c) respectively.

Fig. 2.8. Experimental setup for the synthesis of MBNC.
2.4.3 Morphology and chemical composition

Scanning electron microscopy (SEM) was used to reveal the morphology of the MBNC pellicles. To this purpose, MBNC was freeze-dried and coated with a thin film of gold. The chemical composition of the MBNC was analyzed via XPS.

2.4.4 Magnetic properties measured via vibrating sample magnetometer and magnetic force microscope

A vibrating sample magnetometer (VSM) (Quantum Design, USA) was used for magnetic measurements. Freeze dried MBNC samples were packed into non-soluble gel capsules of 5 mm diameter. The gel capsule was closed with its cap and inserted in the straw as a sample holder. The loaded capsule was contained inside a plastic straw. The loaded straw was placed inside a magnetometer and magnetization was measured at 300 K in an applied field of ± 11000 Oe. The VSM works by measuring the induced voltage in pick up coils as the sample is vibrated sinusoidally inside a magnetic field. Magnetic domains on an air-dried MBNC were revealed using a magnetic force microscope (MFM) (JPK Instruments AG, Germany).

2.4.5. Porosity and swelling ratio

Physical properties such as the porosity and swelling ratio were determined for BNC, using at least 10 replicates per test. The swelling ratio or the amount of water absorbed by BNC was obtained as the ratio of the mass of the swollen pellicle to the mass of the freeze-dried pellicle (see equation (1)) (Gehrke et al, 1997). The porosity was estimated using the Archimedes’ principle (Agrawal, 2002) through equation (2), where \( M_s \) is the BNC’s swollen mass, \( M_d \) is the BNC’s dried mass and \( M_f \) is the measured submerged mass of the BNC in water. The iron content on the MBNC was calculated by subtracting the wet and freeze-dried mass of the BNC before and after of the ferrogel synthesis.

\[
q = \frac{M_s}{M_d} \quad (1)
\]
\[
\% \text{ Porosity} = \frac{M_s - M_d}{M_s - M_f} \quad (2)
\]

2.4.6. Permeability

The permeability of BNC and MBNC was estimated using a horizontal diffusion cell (side-by-side diffusion cell, PermeGear), following the protocol described by Sokolnicki and colleagues (2006). Two marker molecules were used in this test: lysozyme from chicken egg white (14307 Da) and bovine serum albumin (BSA, 66 kDa). The diffusion cell had an orifice diameter 9mm and a volume capacity of 5 ml. The donor chamber was filled with 5 ml of a solution of containing either 10 mg/ml of B12 (1355 Da), 0.5 mg/ml of lysozyme (14307 Da), or 8.2 mg/ml of BSA (66 kDa). All the solutions were prepared in sodium acetate buffer solution (pH 7.0±0.05 at 25 °C, 0.2 µm filtered). The donor chamber was filled with 5 ml of sodium acetate buffer solution free of any marker molecule. Both chambers were stirred at 700 rpm, and the junctions were sealed with vacuum grease. The concentration of the marker molecules at different time points was obtained using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, USA) operated at 280 nm. NanoDrop does not require a standard curve, and only uses 2 µl of volume to determine the concentration of a protein in solution.

The permeability of the different marker molecules across the membrane is obtained from the slope of a plot \(-\ln(\Delta C_t/\Delta C_0)\) versus time, according to the Fick’s second law in a pseudo-steady state (equation (3)) (Cussler 2007; Crank 1975; Gehrke et al. 1997). Here, the \(\Delta C\) denotes the concentration gradient between the two reservoirs at times \(t=0\) and \(t=t\). In pseudo-steady state, the following assumptions are made: (1) the diffusion coefficient (D) and partition coefficient (H) are constant and independent of the reservoir concentration; (2) the flux across the membrane quickly reaches a pseudo-state value even though the concentrations in the donor and receptor chambers are changing with time; (3) the membrane has smaller volume compare to that of the adjacent reservoirs (Cussler 2007).

\[
P = \frac{1}{\beta t} \ln \left( \frac{C^0_d - C^0_r}{C^t_d - C^t_r} \right) \quad (3)
\]
Where

\[
\beta = \frac{A}{l} \left( \frac{1}{V_r} + \frac{1}{V_d} \right)
\]

\(A\) and \(l\) denote the area and thickness of the BNC pellicle respectively, \(t\) is the time in hours, \(C^0\) is the initial concentration, and \(V\) is the reservoir volume. The subscripts \(d\) and \(r\) indicates the donor and receptor reservoirs respectively. **Fig.2.9** shows the experimental setup for a side-by-side diffusion cell.

**Figure 2.9.** Side-by-side diffusion cell: At the beginning of the experiment, the donor chamber (a) is filled with the marker protein while the receptor chamber (b) only contains sodium acetate buffer solution free of any marker molecule. The membrane is placed in between the two reservoirs. A hot plate is used to stir the solutions. The change in concentration over time is measured on both chambers to determine the permeation coefficient using equation (3)
2.5 References


3.1 Introduction

Blood-contacting devices not only have to meet adequate attributes in terms of biocompatibility and hemocompatibility but also, must own suitable mechanical properties able to withstand the blood pulsatile nature. Regarding to these properties, BNC holds remarkable advantages for the substitution of small-diameter vessels, which include: (1) customizable shape, (2) nanofibril network with a large surface area, (3) open-pore structure which can be easily tuned by biosynthetic or chemical means, (4) a compliance resembles that of the native arteries, and (5) biocompatibility (Huang 2014).

BNC can be easily modified to further expand its functionalities, or be used along with other additives to form composites with diverse biomedical and technological applications. For example, silver nanoparticles have been introduced on BNC pellicles to develop recoverable and reusable antibacterial agents (Sureshkumar et al. 2010). Magnetic hydrogels or “magnetic responsive polymer nanocomposites” based on BNC have also been fabricated by the incorporation of cobalt ferrite nanoparticles in the BNC network, with potential uses in microfluidic devices and electronic actuators (Olsson et al. 2010).

Because of the real-time controllable elastic properties of the magnetic hydrogels, those can be used to remotely release drugs-on-demand. Liu and coworkers (2008) designed a magnetic hydrogel by precipitating magnetite on polyvinyl alcohol (PVA). Those researchers found that the permeation rate of vitamin B12 across the material decreased by increasing the intensity of direct current magnetic field at which was exposed the material, mainly because the magnetic nanoparticles within the magnetic hydrogel aggregate together reducing the porosity of the magnetic hydrogel (Liu et al. 2008).

Magnetic hydrogels have also the ability to attract and take up cells, growth factors and other molecules bound to magnetic nanoparticles to sites that are unfavorable for tissue regrowth or
difficult to access by other means (Li et al. 2013). For instance, high hemodynamic stresses at the site of the aneurysmal neck defect limit the rate of reendothelialization, which may take place several weeks after endovascular intervention, because the endothelial cells are continuously washed out from the injured site limiting cell proliferation (Pislaru et al. 2006). Reendothelialization is the process by which endothelial cells form a new intima functional arterial layer with native antithrombotic properties.

To overcome the poor rate of reendothelialization on arterial injuries, cell-based therapies have explored the delivery of endothelial progenitor cells at the damaged arterial sites, but it requires a blood cessation of up to 20 min to increase cell retention (Pislaru et al. 2006). By loading human cells with superparamagnetic iron oxide nanoparticles (SPION), high cell densities have been achieved on deeper sites of three-dimensional porous scaffolds and at the wires of steel stents (Reddy et al. 2012; Polyak et al. 2008). Polyak and colleagues (2008) used a uniform magnetic field to generate high-gradient magnetic fields on the wires of a 304 grade steel stent for the purpose of maximizing the local magnetic force on endothelial cells loaded with magnetic nanoparticles. They achieved local targeting of endothelial cells at the wires of the stent in dynamic fluid flow conditions. The magnetic saturation for this steel stent was about 10 emu g\(^{-1}\) and occurred in a range of 1–2 kGauss. Using a flow-loop system, those authors found that 50% of the captured cells (0.25 x10\(^6\)) accumulated on the stent surface within the first 6 min. Importantly, magnetic hydrogels subject to a uniform magnetic field lead to higher gradient magnetic fields near/inside the material than those reported for magnetized steel stents, by virtue of their ability to alter the distribution of the external uniform magnetic flux (Sensenig et al. 2012).

When a magnetic hydrogel is placed in a uniform magnetic field, the force that acts in the SPION-loaded cells is the superposition of the external uniform magnetic field and the magnetic field gradient due to the magnetized elements inside the magnetic hydrogel. The magnetic hydrogel concentrates and alters the external magnetic field, forming a magnetic field gradient that maximizes the magnetic force that acts on the SPION-loaded cells to locally target them, even if they are far away from the material (Kang et al. 2009). The total magnetic force \(F_{\text{tm}}\) acting on a SPION-loaded cell can be described as the sum of the magnetic forces acting on each magnetic nanoparticle inside the cell, and which is obtained using an effective dipole moment approach as depicted by equation (4) (Kang et al. 2009; Furlani 2007):
\[ F_{tm} = \frac{1}{2\mu_0 N_m (\chi_c - \chi_f)(H_a \cdot \nabla)H_a } \]  (4)

Where \( N_m \) is the number of magnetic nanoparticles inside the cell, \( \chi_c \) and \( \chi_f \) are the susceptibility of the magnetic hydrogel and the surrounding medium respectively, \( H_a \) is the applied magnetic field and it is the sum of the uniform magnetic filed \( H_u \) and the magnetic field gradient \( H_g \) formed by the magnetic hydrogel. This latest depends on \( H_u \) through the magnetic saturation of the material, but the field of \( H_g \) does not influence the field of another (Furlani et al. 2007). Therefore, the magnetic force acting on SPION-loaded cells can be tuned by increasing the external magnetic field up to the maximum magnetic saturation of the material (Kang et al. 2009).

Because deoxygenated red blood cells behave as a paramagnetic material, a magnetic force can act on them in a similar way as SPION-loaded cells. This property is advantages for example, to induce thrombus formation inside the aneurysmal defect and outside of the luminal space. This may increase the blood cessation in and out from the aneurysm, and contribute to a rapid obliteration of the aneurysm from the parent artery.

Based on the above concepts of magnetic separation of SPION-loaded cells and red blood cells, we fabricated and characterized a magnetic hydrogel based on BNC, which we named magnetic bacterial cellulose (MBNC), to magnetically capture circulating cells, and therefore, improve the reendothelization rate at the aneurysmal neck defect. This idea is illustrated in Fig. 3.1. In chapter I, we found values for the MBNC magnetic saturation of up to 10 emu g\(^{-1}\), which is in agreement to those reported by other researchers under similar experimental conditions, and for steel stent materials (10 emu g\(^{-1}\)). The MBNC was prepared by \textit{in situ} precipitation method of Fe\(^{3+}\) and Fe\(^{2+}\) iron salts on BNC pellicles using ammonium hydroxide as precipitating agent. Subsequently, MBNC was coated with dextran to protect the embedded SPION from oxidation and at the same time, improve the biocompatibility of the bare magnetite.
Figure 3.1. Graphic representation of the gradient magnetic field formed on the MBNC as result of uniform magnetic flux exposure. SPION-loaded cells are attracted to the MBNC even though if they are far away from the scaffold. The force acting on the magnetically functionalized cells is the sum of the external magnetic field and the gradient magnetic field created by the MBNC.

In the present chapter, we hypothesized that the MBNC can create a magnetic field gradient to locally target SPION-loaded cells to the MBNC surface, which at the same time, can serve as a cell nidus for cell proliferation. Once the cells are captured, the external magnetic field is removed and the magnetic force acting on the cells vanishes. Additionally, to guarantee that cells remain attached to the MBNC once the external magnetic field is removed, we introduced cell adhesion sites on the surface of the MBNC via collagen-conjugation using CDAP as activator vehicle. This is important because native BNC lacks cell adhesion sites.

We designed a parallel plate-flow chamber to test cell take up and cell retention under pulsatile fluid flow. We also evaluated the MBNC biocompatibility using a life/dead assay. For these experiments, we use four types of MBNC: 100 mM MBNC, 50 mM MBNC, 25 mM MBNC, and collagen-conjugated MBNC (50 mM coll-MBNC). Those samples differ in the concentration of iron.
salts used during the synthesis of the MBNC. For example, 100 mM MBNC refers to 100 mM of Fe$^{3+}$ in the initial iron salt solution.

3.2 Results and Discussion

3.2.1 Biocompatibility of dextran-coated MBNC

We investigated the biocompatibility of dextran-coated MBNC bearing decreasing SPION concentrations by culturing HASMC on direct contact with each sample for 4 days, and then, analyzing the transformation of calcein AM into a fluorescent protein (green), which is only carried out by healthy cells. Results are presented in Fig. 3.2. Apoptotic cells, on the other hand, are permeable to EthD-1 (red) and unable to transform enzymatically calcein AM.

MBNC samples were prepared with 3 different initial concentrations of iron salts, which were named according to the molar concentration of Fe$^{3+}$ in the starting solution: 100 mM, 50 mM and 25 mM MBNC. The magnetic nanoparticles formed inside the MBNC were then coated with dextran to improve the biocompatibility and stability of the embedded SPION. Dextran is a branched polysaccharide commonly used to coat magnetic nanoparticles, owing to its biocompatibility and antithrombotic properties (Qiao et al. 2009). For 30 nm magnetite nanoparticles, it has shown that dextran coating reduces the formation of reactive oxygen species (ROS) and cell toxicity compare to bare magnetite (Yu et al. 2012).

Fig. 3.2 shows that 25 mM MBNC is suitable for cell cultivation as it gives a cell survival percentage similar to untreated BNC, which corresponds to the negative control, at least during 4 days of cell culture (Fig. 3.3). On the other hand, MBNC with the higher SPION concentrations of 50 mM and 100 mM give the poorest biocompatibility under the experimental conditions reported here. These results are summarized in Fig 3.3. Because cell viability and metabolic activity decrease significantly when cells are exposed to high magnetic nanoparticle concentrations (Yu et al. 2012), it is probably that the SPION start releasing very rapidly from the MBNC, so cells were exposed to harmful SPION concentrations. Indeed, SPION are not covalently bound to the BNC network, only by ion-dipole interactions, therefore, they are expected to release from the MBNC overtime.
In vitro studies using a magnetic hydrogel made of Poly(N-isopropylacrylamide) and iron oxide nanoparticles (~3 wt % Fe₃O₄) showed that this nanocomposite did not have adverse effects on NIH 3T3 fibroblast viability as seen by the live cells available on the material after 7 days (Meeach et al. 2009). Moreover, Sapir and coworkers (2012) have reported similar results in terms of biocompatibility for alginate-based magnetic hydrogels (8% (w/v) of final magnetite concentration). Those later researchers evaluated the endothelial cell activity and growth of endothelial cells on alginate impregnated with magnetically responsive nanoparticles (magnetite) and observed low toxicity effects for the analyzed material. They also observed more pronounced cell metabolic activity in the magnetite-loaded alginate exposed to a magnetic field compare to unexposed control samples (Sapir et al. 2012).

Figure 3.2. Fluorescence microscopy images of live (green) and dead (red) human aortic smooth muscle cells (HASMC) on (a) BNC (5X), (b) 25 mM MBNC (5X), (c) 50 mM MBNC (20 X) and (d) 100 mM MBNC (20X). Note: 5X and 20X refers to the image magnification.
Since both poly(N-isopropylacrylamide) and alginate are known to be biocompatible, it is believed they provide a shielding effect to the embedded nanoparticulate material. Moreover, for those nanocomposites, the magnetite concentration was kept low compared to the volume or mass of the hydrogel. This is agreement with the results reported here, where the lowest SPION concentration (50 mM MBNC) gave the most satisfactory biocompatibility in terms of cells viability for at least 4 days of cell cultivation.

![Cytotoxicity of dextran-coated MBNC](image)

**Figure 3.3.** Percentage of live cells normalized by the total number of cells on the tested material.

### 3.2.2 MBNC collagen-conjugation

To guarantee that cells remain attached to the MBNC once the external magnetic field is removed, the MBNC must bear specialized sites for cell adhesion such as the Arg-Gly-Asp (RGD) sequence or other recognition sites for integrins. The RGD sequence is a peptide sequence found several extracellular matrix (ECM) proteins such as fibronectin, collagen, laminin; and acts as a
physical anchoring mechanism between the ECM and the cell via integrin ligand binding (Hersel et al. 2003). Natively, BNC lacks cell recognition motives, and therefore, this may hamper the establishment of a strong cell adhesion to the BNC.

In order to establish a permanent interaction cell/MBNC, the RGD sequence or ECM proteins carrying it, need to be covalently linked to the BNC fibrils. This can be accomplished by introducing intermediate reactive compounds that react with the hydroxyl groups in the BNC backbone. Those intermediate reactive groups are then substituted by strong nucleophiles such as amine, which is present in all protein types (Kuzmenko et al. 2013). Among the activating agents for cellulose, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) is advantageous because the activation reaction is carried out in aqueous solution and takes only a few minutes. CDAP has been used for collagen and fibronectin conjugation to BNC (Kuzmenko et al. 2013), and for activation of cellulose membranes for immunosensor applications (Stöllner et al. 2002).

We used CDAP as an activating vehicle of BNC and MBNC hydroxyl groups following protocol proposed by Stöllner and coworkers. For this, BNC and 50 mM MBNC pellicles of 16 mm in diameter were exposed to a solution of CDAP-triethylamine for 1 min, and then, the reaction was stopped by using either 0.1 N of HCL or 0.1 M acetate buffer (pH 4.6). The BNC and MBNC were subsequently incubated overnight in a collagen solution. We then evaluated the biocompatibility of the collagen-conjugated BNC and MBNC using a live/dead assay as described previously for the case of unconjugated-MBNC. The fluorescence microscopy images are presented in Fig. 3.4.
Figure 3.4. Biocompatibility evaluation of (a) collagen-conjugated BNC and (b) 50 mM collagen-conjugated MBNC using a live/dead assay based on Calcein and ethidium bromide homodimer (EthD-1) fluorescence detection.

We observed detrimental effects for both collagen-conjugated BNC and MBNC in terms of cell viability. This toxicity can stem from unreacted residues and byproducts that remain on the material after hydroxyl activation. This was verified by XPS data, where the presence of fluorine and chlorine were detected (Fig. 3.5-3.6). The relative concentrations of important chemical species are summarized in Table 3.1 and 3.2. The high cell toxicity of collagen-conjugated MBNC can also be due to an additional effect: the release SPION from the MBNC at concentrations that exceed the doses that are safe for cell integrity. The XPS data also shows the presence of nitrogen, which can be interpreted as a successful conjugation of collagen to BNC and MBNC pellicles. The nitrogen concentration was practically zero for BNC and MBNC samples.

To reduce the presence of byproducts after collagen-conjugation, lower amounts of CDAP were used as well as an extensive washing of the materials. We also used acetate buffer (pH=4.6) to stop the activation reaction as an alternative to HCL. After implementing those changes, we did not observe improvements on terms of cell viability.
Table 3.1. Atomic concentration of the chemical elements detected by XPS on collagen-conjugated BNC

<table>
<thead>
<tr>
<th>Sample</th>
<th>C 1s (at.%)</th>
<th>O 1s (at.%)</th>
<th>N 1s (at.%)</th>
<th>F 1s (at.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNC</td>
<td>64.95</td>
<td>35.05</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>BNC + CDAP</td>
<td>63.24</td>
<td>32.42</td>
<td>4.35</td>
<td>--</td>
</tr>
<tr>
<td>BNC + CDAP + Collagen</td>
<td>60.79</td>
<td>35.84</td>
<td>2.43</td>
<td>0.94</td>
</tr>
</tbody>
</table>

**Figure 3.5.** XPS survey for collagen-conjugated BNC. The presence of nitrogen in CDAP-activated BNC (blue) and collagen-conjugated BNC (red) indicates the incorporation of the activator agent and collagen to the BNC surface respectively. Notice that untreated BNC lacks nitrogen and its main constituents are oxygen, carbon and hydrogen (not shown).
Table 3.2. Atomic concentration of the chemical elements detected by XPS on collagen-conjugated MBNC

<table>
<thead>
<tr>
<th>Sample</th>
<th>C 1s (at.%)</th>
<th>O 1s (at.%)</th>
<th>N 1s (at.%)</th>
<th>Fe 2p (at.%)</th>
<th>Cl 2p (at.%)</th>
<th>Na 1s (at.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNC</td>
<td>64.95</td>
<td>35.05</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MBNC</td>
<td>13.95</td>
<td>52.95</td>
<td>--</td>
<td>31.36</td>
<td>1.74</td>
<td>--</td>
</tr>
<tr>
<td>MBNC + CDAP</td>
<td>17.8</td>
<td>47.08</td>
<td>1.3</td>
<td>26.07</td>
<td>6</td>
<td>2.09</td>
</tr>
<tr>
<td>MBNC + CDAP + Collagen</td>
<td>26.62</td>
<td>42.77</td>
<td>6.66</td>
<td>25.74</td>
<td>4.05</td>
<td>1.97</td>
</tr>
</tbody>
</table>

Figure 3.6. XPS survey for collagen-conjugated MBNC. The reduction in intensity of the Fe 2p and Fe 3s bands indicates that the MBNC surface is coated with CDAP and CDAP/Collagen.
3.2.3 Dynamic cell capture test using a parallel-plate flow chamber

To test the hypothesis that MBNC can locally target SPION-loaded cells by creating a strong magnetic field gradient across its surface, we designed a parallel-plate flow system to capture magnetically functionalized circulating cells. We placed this chamber perpendicular to the gravity force, so the effect of cell retention could be only due to the magnetic force applied to the SPION-loaded cells (see experimental section for more details). The results of cell capture by the MBNC with (Mag +) and without (Mag -) an external magnetic field are depicted in Fig. 3.7 and summarized in Fig. 3.8.

To create the external magnetic field, two neodymium magnets with a maximum magnetic strength of 0.3 T measured at its surface, were placed facing one another and in between them, the parallel-plate flow chamber was positioned. The neodymium magnets create a fairly uniform magnetic field that is used to excite the SPION embedded in the MBNC, and therefore, produce a gradient magnetic field across the hydrogel. This gradient magnetic field exerts the force to target SPION-loaded cells to the MBNC surface. We observed higher cell retention for magnetically excited MBNC compared to MBNC without exposure to an external magnetic field (Fig. 3.7). The results were comparable between MBNC with different SPION concentrations, showing that even with the 50 mM MBNC, cell targeting can be achieved.
**Figure 3.7.** Mag + (left) denotes MBNC samples that were placed on an external magnetic field, whereas Mag – (right) indicates absence of an external magnetic field. For Mag +, the MBNC is magnetized, and therefore, it applies an attraction force on magnetically functionalized cells.
Images correspond to MBNC at 3 different SPION concentrations (100 mM, 50 mM and 25 mM) with (Mag +) and without (Mag -) an external magnetic field exposure.

**Figure 3.8.** Number of cells capture on the MBNC under pulsatile flow conditions. Comparisons are made between MBNC with different SPION concentrations and with (Mag +) and without (Mag -) exposure to an external magnetic field.

Even though cell coverage of MBNC was inferior to that observed on magnetized steel stent used by Polyak and coworkers (2008), this discrepancy may stem from: (1) a lower concentration of circulating cells, (2) a shorter time of cell capture, and (3) the pulsatile nature of our fluidic system. Pulsatile fluid flow may result in higher shear stresses at the surface of the MBNC, therefore, the peak wall shear stress might be actually higher than that based on our calculations. However, cell capture was improved when the collagen-conjugated MBNC was employed when using the same fluidic configuration and timing described before (Fig. 3.9).
Because the MBNC is not a completely smooth surface, but instead, it is rough as observed by AFM (Fig. 2.7), there is a high probability of bubble formation, which affects accuracy of our cell capture system and may contribute to cell capture in non-magnetized MBNC (Fig. 3.9).

To best of our knowledge, this is the first work using a magnetic hydrogel based on bacterial cellulose to physically target cells to its surface, and which has been specifically designed for aneurysmal wall reconstruction. Other authors such as Pislaru and coworkers (2006) used permanent magnets that were applied on the outside surface of a vascular graft (Dacron) to rapidly capture and retain endothelial cells on the prosthetic material. Even though Pislaru’s approach was able to magnetically capture cells, which is especially crucial for speed up the re-endothelization in small diameter arteries, the magnetic material used in this approach was not biocompatible, and thus, no suitable for long-term experiments.

3.3 Concluding remarks

A cerebral aneurysm is a life-threatening disease that requires a prompt and complete obliteration from the parent artery to avoid its rupture. A potential way to speed up aneurysmal closure and occlusion is to provide rapid endothelial coverage across of the aneurysmal neck defect (Pislaru et al. 2006). Endothelial cells form a passive luminal layer that prevents the damaged site
from restenosis. In the present work, we hypothesized that a magnetic hydrogel based on bacterial nanocellulose could be used as cell capture and retention platform to expedite reendothelization. To test the feasibility of our approach, we fabricated a magnetic BNC or MBNC and investigated its physical and magnetic properties. We then evaluated the MBNC in terms of biocompatibility. We found that low SPION concentrations in the MBNC (50 mM) yielded the higher smooth muscle cell survival. Even at this low concentration of SPION, MBNC could capture circulating HASMC, which demonstrated our hypothesis. Even though we did not use endothelial cells, our results represent a proof-of-concept and can be used to capture any type of cells for another type of application.

3.4 Materials and methods

3.4.1 Collagen-conjugation of BNC and MBNC membranes

Note: All the reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

A stock solution of 50 mg/ml of 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) was prepared by dissolving 50 mg of CDAP in 1 ml of acetonitrile and stored at −20 °C (Stollner et al, 2002). Using a small vial, 400 ul of the CDAP stock solution were combined with 400 ul of 0.2 M of triethylamine (TEA) and mixed well. Never dried BNC and 50 mM MBNC pellicles of 16 mm in diameter were wiped dry, and then 100 ul of the CDAP-TEA solution was added on top of each pellicle and let react for 1 min. Immediately after, either 1 ml of 0.1 M acetate buffer (pH 4.6) was used to stop the reaction. The pellicles were rinsed several times with sterile high purity water and vortex mixed to remove excess of acetate buffer. BNC and MBNC pellicles were subsequently transferred to a solution of collagen (200 ul of collagen stock solution in 2 ml of sterile high purity water), and left to react overnight at 2 °C (Kuzmenko et al, 2013). Before use, the samples were equilibrated in phosphate buffer saline.
3.4.2. Chemical composition

Collagen-conjugated BNC and MBNC pellicles were allowed to air-dried and then subject to chemical composition survey via X-ray photoelectron spectroscopy (XPS). The samples were analyzed for the presence of collagen, and residues of by products such as CDAP, chlorine and acetate buffer.

3.4.3. Biocompatibility

Human aortic smooth muscle cells (HASMC) (Thermofisher Scientific Inc, USA) were cultured on BNC, MBNC, and collagen-bioconjugated BNC and MBNC pellicles for 4 days according to the manufacture’s instructions (Thermofisher Scientific, USA). After that, a live/dead assay based on calcein AM and ethidium homodimer (EthD-1) fluorescent detection was performed to determine the cell viability of HASMC on the samples (Thermofisher Scientific, USA). Healthy and viable cells transform calcein by enzymatic conversion into a fluorescent protein, thereby producing bright green color. On the other hand, apoptotic cells are unable to transform calcein, however are permeable to EthD-1 and marked in red color. Samples were imaged using an epifluorescence microscope and the number of viable cells was average over the total number of cells in each frame image taken at different points across the tested materials.

3.4.4. Parallel plate-flow chamber design

To evaluate the cell take up under dynamic flow conditions, a close-loop fluidic system was designed consisting of a parallel plate-flow chamber, a peristaltic pump, and tubing (Fig. 6). The chamber consists of a circular base of 16 mm of internal diameter, a silicone rubber gasket to create the geometry for the fluid flow, and a tubing adaptor (Fig. 3.10 (a)). Those elements are assembled together in Fig. 3.10 (b), along with the flow system set-up in Fig. 3.10 (c). In this fluidic system, the shear stress can be adjusted by changing the thickness of the gasket according to equation (5), where \( \tau \) is the shear stress, \( w \) is the width and \( h \) height of gasket’s opening respectively, \( \mu \) is the viscosity, and \( Q \) is the flow rate.
\[
\tau = \frac{6\mu Q}{wh^2} \quad (5)
\]

The typical arterial shear stress is about 15 dynes cm\(^2\), and the viscosity of the phosphate-buffer saline is equal to 1.05cP (0.0105 g cm\(^{-1}\) s\(^{-1}\)) at room temperature. We used a silicone rubber gasket with a thickness of 1.5 mm and a rectangular opening 5x10 mm\(^2\). The maximum flow rate for our peristaltic pump is 6.5 ml/min, which give us a shear stress of 0.514 dynes cm\(^2\). Even though this shear stress is lower than the typical arterial shear stress, it is still relevant for the analysis of cell retention under fluid stress and magnetic field exposure.

**Figure 3.10.** (a) Pieces forming the parallel-plate flow chamber, (b) assembled chamber, and (c) flow system set-up.
3.4.5 Magnetic functionalization of HASMC

HASMC were magnetically functionalized with magnetic nanoparticles by passive uptake. Briefly, HASMC were cultured on 6-well plate according to the manufacture's directions (Thermofisher Scientific, USA). Dextran-coated nanoparticles of 30 nm in diameter at a concentration of 0.1 mg ml\(^{-1}\) were added to the culture medium to for 24 hours.

3.4.6 Cell capture

The MBNC ability to attract and retain magnetically functionalized cells in flow conditions were tested by exposing the magnetized MBNC to a circulating suspension of SPION-loaded HASMC at a flow rate of 0.514 dynes cm\(^{-2}\). To magnetize the MBNC, a external magnetic field \(H_u\) was created by placing two magnets facing each other and separated by the parallel-plate flow chamber, with a maximum magnetic strength of 0.3 Tesla measured at the surface of each magnet (Fig. 3.11 (a)). The MBNC distorts \(H_u\) and creates a magnetic field gradient, which exerts a magnetic force on the SPION-loaded cells that is perpendicular to the gravity force (Fig. 3.11 (b)). Therefore, the cells capture by the MBNC are due mainly to the magnetic force applied on them.

Four types of MBNC were tested using the fluidic system in Fig. 3.10 and configuration in Fig. 3.11: three MBNC samples with decreasing concentration of SPION (100 mM, 50 mM and 25 mM), and a MBNC sample that was collagen-bioconjugated (50 mM coll-MBNC). For each run, a freshly prepared SPION-loaded HASMC was used, which was fluorescently labeled with calcein AM (Thermofisher Scientific, USA). Those cells were circulated through the fluidic system for 15 min, and after that, the MBNC was removed and the fluid flow path created by the gasket was imaged using an epifluorescent microscope (Fig. 3.12). Cell capture was quantified by averaging the number of HASMC cells along the gasket opening, except at the inlet and outlet orifices.
Figure 3.11. SPION-loaded HASMC are taken up due to the magnetic field gradient created by the MBNC when it is placed in an external magnetic field $H_u$.

Figure 3.12. A gasket made of silicone rubber with a rectangular opening forms the path for fluid flow in the parallel-plate flow chamber. After circulating the SPION-loaded HASMC in the fluidic system for 15 min, the MBNC is removed and imaged along the gasket opening.
3.5 References


Stöllner, D., Scheller, F. W., & Warsinke, A. (2002). Activation of cellulose membranes with 1, 1′-carbonyldiimidazole or 1-cyano-4-dimethylaminopyridinium tetrafluoroborate as a basis for the development of immunosensors. Analytical biochemistry, 304(2), 157-165.

CHAPTER 4
FUTURE DIRECTIONS

4.1 Introduction

Bacterial nanocellulose (BNC) possesses advantageous properties for vascular replacement applications because its structure and mechanical properties resemble that of the native arteries. It has been shown that BNC induces a low thrombogenic response in terms of platelet consumption when it is compared to the most commonly used synthetic polymers for vascular grafts (e.g. poly(ethylene terephthalate (PET)) and poly(tetra fluoroethylene)(ePTFE)). However, the BNC has need of inducing endothelization to reduce complement system activation if used as a vascular graft (Fink et al. 2010).

The BNC also has a nanofibril structure with a large surface area and abundant reactive hydroxyl groups, which cause strong electrostatic interactions with metallic cations and compounds capable of hydrogen-bond formation (Klemm et al. 2011). In fact, this property has been explored to design advanced polymeric materials and nanocomposites with improved biological, optical, mechanical, and magnetic properties (de Santa Maria et al. 2009; Nogi et al. 2008; Feng et al. 2012; Sureshkumar et al. 2010). For example, de Santa Maria and coworkers (2009) impregnated silver nanoparticles into BNC through a chemical reduction process, in which the BNC was immersed into a silver nitrate solution and then \textit{in situ} \(\text{Ag}^+\) reduction was performed by using either hydrazine, hydroxylamine, or ascorbic acid. Because the antimicrobial activity of the silver nanoparticles, this nanocomposite has the potential to be used as an antimicrobial wound dressing for chronic wounds and burns.

Even though the BNC can be used as a template for the nucleation of different types of nanoparticles, the anchor of those to the BNC fibrils tend to be weak. We observed that BNC carrying high SPION concentrations had pronounced detrimental effects on human smooth muscle cell viability, presumably because SPION were released very rapidly from the membrane. This later was observed as darkening of the cell culture medium where the magnetic BNC (MBNC) was placed, even though extensive washing of the MBNC was performed previously to biological testing.
Weak binding of nanoparticles to BNC fibrils was also reported by de Santa Maria and coworkers (2009), in the case of silver nanoparticles embedded on BNC.

To induce a stronger attachment of silver nanoparticles to the BNC, Ifuku and coworkers (2009) introduced carboxylate groups into cellulose by a catalytic oxidation process using 2,2,6,6-tetramethylpyperidine-1-oxy radical (TEMPO). Carboxylate groups can be used to host Ag\(^+\) ions by an ion-exchange reaction, after which silver nanoparticles are formed by a reduction reaction (Ifuku et al. 2009). More recently, Wu and coworkers (2014) proposed another chemical route improving silver nanoparticle’s attachment and release to and from the BNC respectively, by using a reaction based on Tollens' reagent.

Stronger adsorption of nanoparticles to the BNC nanofibrils can also be achieved by using plasma treatment. Surface activation via plasma treatment has been used to create reactive groups on synthetic polymers such as poly(ethylene terephthalate) (PET) using heavy ions (Ar\(^{+9}\)) (Hersel et al. 2003). Similarly, oxygen plasma treatment of cellulose fabrics has been used to improve the adsorption of chitosan by inducing the formation of functional groups such as C-O, CdO, and O-CdO along the cellulose nanofibril structure (Fras Zemljic et al. 2009).

We used collagen-conjugation of the MBNC via 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) activation to incorporate cell adhesion sites and guarantee that the cells remain attached to the MBNC once the external magnetic field was removed and the magnetic force vanished. Our results showed that collagen increased cell coverage, indicating that once the MBNC magnetic force vanished, cells required recognition motives to remain attached to the MBNC surface. However, we observed suboptimal results in terms of cell viability after four days of cell culture for CDAP-activated BNC and MBNC due probably to residual byproducts that were highly toxic (e.g. fluorine).

An alternative route for collagen-conjugation is to active the MBNC’s hydroxyl groups by using N, N’-disuccinimidyl carbonate, tresyl chloride or p-nitrophenyl chlorocarbonate (Hersel et al. 2003). Moreover, minimal essential cell adhesion peptide sequences, i.e. RGD sequence, can be used instead collagen to improve cell adhesion specificity.
Additionally, we used smooth muscle cells in our experiments as a proof-of-concept, however, further studies require analyzing cell survival and functionality after cell capture using endothelial cells as the real application demands. Immunological response studies need also been conducted on MBNC to get knowledge about how the body could response to this new type of material.

4.2 Conclusion

Magnetic hydrogels represent a new class of advanced functional materials with great potential in tissue engineering and pharmaceutical applications because they can provide a platform to (1) release drugs on demand, (2) speed up reendothelization by increasing cell coverage and survival on damaged arterial areas that otherwise are aggressive for cell proliferation, (3) take up magnetically-functionalized hormones and growth factors, and (4) provide mechanical cues to cells by virtue of its deformability capabilities under an alternating magnetic field (Li et al. 2013; Meenach et al. 2009)

In the present work, we showed that MBNC was able to capture SPION-loaded smooth muscle cells under pulsatile flow conditions even with a low SPION concentration, indicating that a magnetic hydrogel can be designed to magnetically capture cells and still be safe in terms of cell viability. A magnetic hydrogel with these characteristics is advantageous because it can induce high-density cell coverage on sites that are aggressive for cell survival such as the aneurysmal neck defect, and simultaneously create a passive and functional endothelial cell layer that protects the MBNC and aneurysm from restenosis and rupture respectively.
4.3 References


