The Potential of TEMPO-Oxidized Nanofibrillar Cellulose Beads for Cell Delivery Applications

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1 Abstract

2 The advanced development of cell carriers for regenerative medicine and cell therapy demands
3 materials able to sustain cell viability prior to their delivery to the target tissue, ability which can
4 be controlled *via* the shape/size and degradability of the matrix. TEMPO-oxidized nanofibrillar
5 cellulose (ToNFC) macromolecules are negatively charged and therefore can be easily formulated
6 by ionotropic gelation into beads of varying sizes that can release their payload through an
7 erosion-controlled process. We report here for the first time on the preparation of ToNFC beads
8 *via* ionic gelation using CaCl₂ and on their loading with OSTEO-1 rat bone cells, with a view to
9 examine their capacity of sustaining the cell viability and of releasing the bone cells in a controlled
10 manner. The initial results obtained demonstrate that ToNFC is able to protect the OSTEO-1 cells
11 and to maintain their viability for at least two weeks; following gradual disintegration of the beads,
12 a significant cell release and subsequent proliferation was observed after 7 days. These results
13 indicate the considerable potential of nanofibrillar cellulose (ToNFC) for applications in cell
14 therapy and regenerative medicine.

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17 ***Key words:*** *cell carriers, TEMPO oxidized cellulose nanofibers, cell delivery,*
18 *regenerative medicine, bone cells*

35 **Introduction**

36 Tissue engineering platforms designed for regenerative medicine aim to
37 improve cell viability and engraftment into the damaged organ areas and to promote
38 neoformation of functional tissue. Over the past decade there has been a
39 considerable interest in cell-biomaterial constructs capable to provide an
40 appropriate microenvironment for successful cell transplantation, differentiation
41 and improved cellular functionality. Cell replacement therapy for attaining
42 functional repair requires a biocompatible and biodegradable delivery vehicle
43 capable of successfully maintaining cell viability during transport and of
44 appropriately controlling their release.(Ratner et al. 2014)

45 Several promising novel biomaterial platforms have been developed recently,
46 with natural biomaterials such as proteins or polysaccharides attracting increasing
47 attention for the development of cell carrier materials as many of them are natural
48 components of the extracellular matrix and therefore tend to be biocompatible.
49 However, they also have significant disadvantages such as strict purification
50 requirements to avoid immunogenic responses and inherent source-dependent
51 variability (Willerth et al. 2013); polymers such as fibrin,(Wu et al. 2012)
52 alginate,(Bidarra et al. 2011) or gelatin,(Santoro et al. 2014) represent some of the
53 most studied examples.

54 Among the biomaterials that demonstrate potential for tissue engineering and
55 cell delivery applications, plant-derived materials such as cellulose have received
56 considerable less attention. Cellulose is a biocompatible hydrophilic natural
57 polymer composed solely of glucose monomers and has good mechanical
58 properties (Klemm et al. 2005, Gandini 2011); though it is widely employed in
59 many pharmaceutical/medical applications (native or modified as carboxymethyl
60 cellulose or its ether / ester derivatives), its extremely slow degradation within the
61 human body represents a severe limitation for more complex biomedical
62 applications.

63 Nanofibrillar cellulose (NFC) has attracted however a lot of attention recently
64 due to its high affinity to water and its capability to form highly viscous dispersions
65 at very low solid content; NFC shows particular promise due to its well-defined
66 single component structure and the possibility to finely tune its hydrogel
67 properties.(Bhattacharya et al. 2012, Lin et al. 2014) NFC can be conveniently

68 prepared from cellulose using different methods, which lead to nanofibrillar
69 materials with different structural features and properties. One of the alternative
70 methods of separating the native cellulose fibres into nanofibrils is *via* the
71 controlled 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation of
72 the glucose units,(Saito et al. 2007) which generates a cellulose derivative (ToNFC)
73 with increased water solubility due to the high degree of substitution with
74 carboxylic groups (about 90 %). ToNFC has been investigated for the development
75 of flexible, transparent and biodegradable materials with improved mechanical
76 properties,(Fukuzumi et al. 2009, Jradi et al. 2012) however its potential for
77 applications in the biomedical field (such as in drug delivery) is just starting to be
78 explored.(Weishaupt et al. 2015) What makes ToNFC interesting is that
79 carboxymethyl cellulose derivatives with similar structures have been shown to be
80 resorbable in humans; at physiological pH (~7.4), about 90 % wt of oxidized
81 cellulose is solubilized within 21 days and converted into the sodium salt of
82 polyglucuronic acid,(Singh et al. 1982) which is then easily eliminated from the
83 body. In ToNFC, the surface of the nanofibrils is negatively charged so a
84 transparent hydrogel made of individual cellulose nanofibers (3-4 nm width, and a
85 few microns length) can be formed.(Saito 2007)

86 Rationalized by the ability of the carboxylic groups present at the surface of
87 the ToNFC nanofibrils to induce their self-assemblage into beads of various sizes
88 based on electrostatic interactions created in the presence of added counter-ions, we
89 report here for the first time on the preparation of OSTE0-1 cell/ToNFC systems.
90 Results of preliminary *in vitro* investigations of ToNFC beads (particles)
91 formulated as cell carriers that can encapsulate, deliver and then release viable cells
92 for potential tissue engineering applications are described.

93 **Materials and methods**

94 Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS)
95 were purchased from Cultilab (Brazil). The 3-(4,5-dimethylthiazol- 2-yl)-2,5-
96 diphenyltetrazolium salt (MTT) was acquired from Merck (US). Isopropyl alcohol
97 dimethyl sulfoxide and phosphate buffer saline (PBS) were purchased from Synth
98 (Brazil); trypsin was sourced from Gibco (US) and 4',6-Diamidino-2-phenylindole
99 (DAPI) from Sigma (Brazil?). All solvents and reagents (analytical grade) were
100 used as purchased.

101 *ToNFC preparation.* Bleached sugar cane bagasse suspension (10 g, 1 % wt),
102 sodium bromide (1 mmol/g cellulose) and TEMPO reagent (0.1 mmol/g) were
103 stirred together, followed by the slow addition of sodium hypochlorite (NaClO) (5
104 mmol/g cellulose). The pH of the suspension was adjusted to 10 by adding 0.1 M
105 NaOH. The pulp was then washed with deionized water to neutral pH. The oxidized
106 fibers were sonicated in an ultrasonicator (Hielscher, UP 400S, 400W, 24kHz) until
107 the formation of a transparent gel (about 10min). The carboxylic content was
108 determined by conductometric titration.(Araki et al. 2001) The chemical
109 modification was confirmed by FTIR using a Perkin-Elmer Spectrum 100 FT-IR
110 Spectrometer equipped with a single horizontal Golden Gate ATR cell (4 cm⁻¹
111 resolution, 32 scans).

112 *Bone cell culture.* Osteogenic cells from the lineage OSTEO-1 (rat calvarium)
113 were incubated in DMEM culture medium supplemented with fetal bovine serum
114 (10 % v/v) and antibiotics (penicillin 100 U/mL; streptomycin 0.1 mg/mL). The
115 cells were incubated at 37 ± 2 °C in 5% CO₂ atmosphere until they reached 80 to
116 90 % confluence, when they were used for the preparation of the beads.

117 *Preparation of ToNFC/cell system (beads).* All materials were sterilized in an
118 autoclave at 121 °C for 15 min. For the preparation of the beads, 1 mL of ToNFC
119 (0.8 % w/v) was gently mixed with the cells pellet obtained by centrifugation
120 (2.5×10⁴ cells). The beads were prepared by drop wise addition of 15 µL of ToNFC
121 plus cells mix into a 1.2 % (w/v) CaCl₂ solution (50 mL), under magnetic stirring.
122 The beads were separated by filtration using a sieve and used for further
123 measurements as described below.

124 *Cell viability measurements.* Beads (prepared and isolated as described above)
125 were immersed in the wells of a 96 well microplate filled with DMEM culture
126 medium (two beads/well, each bead was about 5 mg) and incubated at 37 °C in a 5
127 % CO₂ atmosphere. The culture media was changed every three days to avoid
128 saturation with cell metabolism products. At each time point (3, 7, 10, 12 and 14
129 day, respectively), the ToNFC beads with encapsulated cells were withdrawn and
130 cell viability tests (MTT assay) were carried out for the supernatant media left in
131 each well after the partially eroded beads where removed. MTT reagent (50 µL; 0,5
132 mg/mL in PBS) was added to each well and the microplates were protected from
133 light and incubated at 37 ± 2 °C for 4 h. The obtained formazan crystals were

134 solubilized in isopropyl alcohol (100 μ L per well) and the absorbance for each well
135 was read at 570 nm in an ELISA microplate reader Polaris-Celer
136 spectrophotometer. The viability assays were performed in three independent runs,
137 in different days, each run being done in triplicate. The cell viability was reported
138 as optical density (O.D.).

139 *SEM studies.* The morphology of the released (adhered) cells was assessed
140 using a FEI (XL50, Philips) Scanning Electron Microscope. Cells were fixed with
141 glutaraldehyde solution (2.5 %) for 40 min at 8 °C, then washed with distilled water,
142 freeze-dried (Liotop L 101) and coated with evaporated carbon in a sputter (Q150R
143 Quorum Technologies, UK) before being examined by SEM. Optical microscopy
144 was carried out using a Leica DM 750 digital microscope equipped with a
145 fluorescence accessory (Hg lamp). For examining the cells, the beads were
146 immersed into 1 μ m/mL DAPI solution for ten minutes and then were analysed by
147 fluorescence microscopy.

148 **Results and discussion**

149 *Preparation of the ToNFC beads.* ToNFC was synthesised via a controlled
150 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation of the C6
151 primary hydroxyl groups of the glucose units, following a previously reported
152 method (Saito 2007). The chemical modification of the cellulosic substrate was
153 confirmed by FTIR-ATR (results presented as supplementary information). The
154 content of carboxylic groups was found to be 1.1 mmol g⁻¹, as determined by
155 conductometric titration. While ToNFC is normally used as a film or gel, here we
156 investigated the assembly of ToNFC as beads in the presence of Ca²⁺ ions (Figure
157 1A) via an ionic gelation process typical for anionic polymers such as alginates
158 (Ostberg et al. 1994); to the best of our knowledge, this is the first report on the
159 preparation of ToNFC beads. The size of the beads can be controlled during the
160 preparation stage by varying the volume of the ToNFC suspension added (Figure
161 1B); beads of about 2 mm in diameter were used for further experiments.

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165 **Figure 1.** (A) A schematic representation of the electrostatically-crosslinked network generated as
 166 a result of the strong interactions between ToNFC carboxylic groups and Ca^{2+} ions. (B) An image
 167 of ToNFC beads of different diameter, obtained using either 100 μL , 50 μL , or 15 μL of ToNFC
 168 suspension (0,8 % w/v), respectively (left to right).

169 *Cell encapsulation into ToNFC.* The incorporation of rat bone cells within the
 170 ToNFC beads was successfully carried out as above, using a suspension composed
 171 of cells and ToNFC instead of just ToNFC solution. Optical microscopy images of
 172 ToNFC beads **with encapsulated** cells are presented in Figure 2, where the cells
 173 located close to the surface are clearly visible (Fig. 2 **A**) and **their presence**
 174 **confirmed by nucleus staining with DAPI (Fig. 2B).**

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Figure 2. (A) ToNFC beads with encapsulated cells indicated by arrows; (B) cell presence confirmed by DAPI staining (blue dichroic filter).

Cell viability. Measurements of cell viability were carried out using an MTT assay that is indicative of the mitochondrion dehydrogenase activity (MTT is reduced by NADH to a purple colored formazan salt that act as a quantifiable marker for living cells only). Results expressed as O.D. (Figure 3) indicated no cell proliferation for at least one week as the result of their protective entrapment within the ToNFC based matrix. At the 7th day time point, the results of MTT indicated an

186 O.D. value of 0.048 (± 0.00), which increased dramatically reaching 1.01 (± 0.20)
187 at day 14.

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189 **Figure 3** - Results of MTT tests indicative of the released cell viability.

190 In addition to the positive MTT results, at the 7 day time point it was possible
191 to visualise sparse cells that became adhered to the bottom of the microplate well
192 (Figure 4 A). The morphology of the adhered cells indicated their spreading,
193 indicative as well of their viability and in agreement with the MTT results (the low
194 number of adhered cells indicated they had just started to proliferate). After 14 days,
195 a confluent cell monolayer with polygonal morphology was observed at the bottom
196 of the microplate wells (Fig. 4 B). It is important to note that the cells were
197 increasingly released into the medium following the gradual erosion/disintegration
198 of the beads. Their slow release was noticeable from day 7 and eventually led to the
199 increased viability detected by MTT at day 14, when most beads were completely
200 fragmented (with the released cells capable to adhere to the bottom of the
201 microplate and even to start proliferating).

203 **Figure 4** – Images of cell adhered at the bottom of the microplate wells: (A) after 7 days; (B) after
204 14 days.

205 SEM images of the cells adhered at the surface of ToNFC after 24 h of
206 encapsulation are presented in Figure 5. The arrows in Figure 5B point to cells
207 present at the surface of ToNFC and the magnified image in Figure 5D shows the
208 morphology of a surface spread cell (Fig. 5C), indicating its viability in contact with
209 ToNFC.

Figure 5. SEM images of ToNFC beads with and without OSTEO-1 cells after 24 h incubation time: (A) no cells; (B, C and D) with cells (different magnification).

Erosion and degradation of ToNFC beads.

Important limitations of typical cellulose materials investigated for biomedical applications are their insolubility and the lack of biodegradation in the human body, explained by the absence of specific enzymes required for this process (cellulases). (Lin 2014) In the case of our ToNFC beads, it appears the degradation observed is a physical process controlled by the gradual loss of Ca^{2+} ions due to their diffusion into the aqueous medium into which the beads were incubated, leading to a Ca^{2+} ion concentration gradient. The time the beads take to disintegrate is an important parameter, as in such applications the carrier material has to deliver the cells well into the target tissue in order to increase the amount of cells required to effect the anticipated regeneration and decrease the healing time. The experiments showed that cells started to adhere to the plate at day 7, indicating

227 that beads needed about a week to noticeably begin disintegrating (leading to the
228 formation of small debris in suspension) and releasing the cells. The results
229 emphasize important advantages of ToNFC compared to unmodified cellulose,
230 namely the possibility to form electrostatically cross-linked hydrogels with better
231 mechanical properties and significantly higher capacity to degrade.

232 **Conclusions**

233 ToNFC was formulated *via* ionic gelation with Ca^{2+} ions into beads that were
234 loaded with OSTEO-1 cells; the size of the ToNFC beads and the cell content can
235 be easily controlled at the preparation stage. The hydrophilic nature of the ToNFC
236 matrix allowed water and nutrient access to the OSTEO-1 cell payload and
237 contributed to maintaining cell viability, as demonstrated by the fast proliferation
238 observed following the release. We believe that the potential to maintain cell
239 viability and to release the cell cargo by physically-induced degradation
240 demonstrated by the TEMPO-oxidized cellulose fibres that were formulated into
241 beads *via* ionic gelation warrants further detailed investigations.

242

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282 **Supplementary Information**

283 The chemical modification of the cellulosic substrate was confirmed by FTIR-ATR, as showed in
 284 Figure S1 for nanofibers of cellulose obtained from sugar cane bagasse (NFC) and for ToNFC. For
 285 both samples, the FTIR spectra revealed the typical features of cellulosic substrates, with strong
 286 bands at around 3300, 2880, and 1100 cm^{-1} associated with the vibrations of the O-H, C-H, and
 287 C-O-C groups, respectively. ToNFC spectra showed the strong peak at 1596 cm^{-1} attributed to the
 288 asymmetric stretching vibration of COO^- , thus confirming the chemical modification of cellulose.

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291 Figure S1. FTIR-ATR spectra of sugar cane nanofibers and its derivative ToNFC.

292 The nanometric dimension of ToNFC as observed by SEM (Figure 2) also supports the intended
 293 chemical modification of the sugar cane bagasse.

Figure S2. SEM image of the surface of freeze dried ToNFC (no cells).

Additional optical microscopic images of empty beads and beads with encapsulated cells are showed in Figure S3. Images of beads with encapsulated cells before and after staining with DAPI are presented in Figure S4.

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 311 Figure S3. (A) Empty ToNFC beads; (B) ToNFC beads with encapsulated cells indicated by arrows,
 312 scale bar 50 μm; (C) ToNFC beads with encapsulated cells indicated by arrows, scale bar 25 μm.
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315 Figure S4. ToNFC beads with encapsulated cells before (A) and after staining with DAPI (B). Scale
 316 bars 50 μm .

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