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1 **Cyclic nigerosyl-1,6-nigerose-based nanosponges: an innovative pH and time-controlled**
2 **nanocarrier for improving cancer treatment**

3
4 F. Caldera^{1*}, M. Argenziano^{2*}, F. Trotta¹, C. Dianzani², L. Gigliotti³, M. Tannous¹, L. Pastero⁴, D.
5 Aquilano⁴, T. Nishimoto⁵, T. Higashiyama⁶, R. Cavalli²

6
7
8
9 ¹Dipartimento di Chimica – Università di Torino. Via P. Giuria 7, 10125 Torino – Italy

10 ²Dipartimento di Scienza e Tecnologia del Farmaco – Università di Torino. Via P. Giuria 9, 10125
11 Torino – Italy

12 ³ Dipartimento di Scienze della Salute, UPO, via Solaroli 17, 28100 Novara - Italy

13 ⁴Dipartimento di Scienze della Terra, Università di Torino. Via V. Caluso 35, 10125 Torino – Italy

14 ⁵Division Manager New Material Development Division R&D Center Hayashibara Co., Ltd. 675-1
15 Fujisaki, Naka-ku, Okayama 702-8006, Japan

16 ⁶NAGASE (EUROPA) GmbH/ Hayashibara - Immermannstrasse 65c, 40210 Düsseldorf, Germany

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21
22 * Equal contribution to the work.

23
24 **Corresponding author:**

25 Roberta Cavalli,
26 Department of Drug Science and Technology,
27 University of Turin
28 Via Pietro Giuria 9,
29 10125 Turin, Italy
30 Phone: +390116707190
31 Fax: +390116707687
32 e-mail: roberta.cavalli@unito.it

33 **Abstract**

34 The design and structural optimisation of a novel polysaccharide-based nanomaterial for the
35 controlled and sustained release of doxorubicin are here reported. A cross-linked polymer was
36 obtained by reacting a tetraglucose, named cyclic nigerosyl-1-6-nigerose (CNN), with pyromellitic
37 dianhydride. The cross-linking reaction formed solid nanoparticles, named nanosponges, able to
38 swell as a function of the pH. Nanoparticle sizes were reduced using High Pressure
39 Homogenization, to obtain uniform nanosuspensions. Doxorubicin was incorporated into the CNN-
40 nanosponges in a good extent. DSC and solid state NMR analyses proved the drug interaction with
41 the polymer matrix. *In vitro* studies demonstrated pH-dependent slow and prolonged release
42 kinetics of the drug from the nanoformulation. Doxorubicin-loaded CNN-nanosponges were easily
43 internalized in A2780 cell line. They might considered an intracellular doxorubicin reservoir, able
44 to slowly release the drug over time. CNN-nanosponges may be promising biocompatible
45 nanocarriers for the sustained delivery of doxorubicin with potential localised application in cancer
46 treatments.

47

48

49 **Keywords:** CNN, nigerose, nanosponges, doxorubicin, sustained release

50

51 **1. Introduction**

52 Controlled and sustained release delivery systems of drugs may open up new avenues in
53 nanotherapeutic fields to overcome some drug limitations, with spatial and time-controlled release
54 kinetics (Arpicco et al., 2016, Prasad et al., 2018). A number of these formulations have used
55 nanoscaled carriers encapsulation. Indeed, drug delivery system-based nanoparticles, able to store
56 and release molecules for an extended period of time (weeks or months) and as a function of an
57 external stimulus, can play a key role in developing safer and more effective nanomedicines (Mura,
58 Nicolas & Couvreur, 2013). The use of nanocarriers allows for the modulation and modification of
59 the physico-chemical properties of drugs, producing improved pharmacokinetics and bio-
60 distribution profiles (Hamidi, Azadi, Rafiei & Ashrafi, 2013). Various biodegradable and
61 biocompatible polymer systems could be used as interesting advanced controlled-release systems
62 for bioactive molecules (Duchene, Cavalli & Gref, 2016). Polymer nanoparticles are easy to
63 produce with improved stability and more control over drug release (Jaimes-Aguirre, et al., 2016).
64 Indeed, drug release kinetics can be precisely controlled by the physico-chemical properties of the
65 polymer, such as molecular weight, porosity, hydrophobicity and crystallinity (Bhattacharjee et al.,
66 2016). Moreover, the presence of specific moiety in the polymer structure, responsive to external
67 stimuli, i.e. dissociable carboxylic groups or redox reactive groups, may facilitate localised drug
68 delivery (Liu, Yang & Urban, 2017).

69 Notably, prolonged-release polymer nanoparticles can reduce the frequency of administration of
70 drugs, particularly those with a short half-life, stabilise drug absorption, decrease the occurrence of
71 adverse side effects and improve patients' adherence to therapy (Natarajan, Nugraha, Ng &
72 Venkatraman, 2014). In particular, poly(lactic-co-glycolic acid (PLGA), a biodegradable polyester
73 approved for human use, has been extensively studied to obtain sustained release nanovectors.
74 Much attention in research has been focused on designing PLGA nanosystems for the controlled
75 delivery of anticancer drugs (Dinarvand, Sepehri, Manoochehri, Rouhani & Atyabi, 2011, Khan et
76 al., 2016). A number of PLGA nanoparticles were studied for doxorubicin, tuning the formulation
77 design to obtain prolonged release kinetics. Doxorubicin-loaded PLGA nanoparticles with the
78 surface modified with poly(L- γ -glutamic acid) (γ -PGA) and finally conjugated with folic acid
79 showed a release profile lasting over 7 days (Jaimes-Aguirre et al., 2017).

80 Cross-linked polymer nanoparticles represent an alternative formulation approach. Recently,
81 cyclodextrin-based nanosponges, hyper cross-linked cyclodextrin polymers, with sizes in the
82 nanometer order of magnitude, have been designed as a drug delivery nanosystem (Trotta, Zanetti
83 & Cavalli, 2012, Liang et al., 2013, Trotta, Dianzani, Caldera, Mognetti & Cavalli, 2014, Caldera,

84 Tannous, Cavalli, Zanetti & Trotta, 2017, Liang et al., 2017, Sherje, Dravyakar, Kadam, Jadhav,
 85 2017). Cyclodextrins are a class of cyclic glucopyranose oligomers with a characteristic toroidal
 86 shape that forms a well-defined truncated cone-shaped lipophilic cavity. Cyclodextrins are able to
 87 include compounds whose geometry and polarity are compatible with that of their cavity
 88 (Muankaew & Loftsson, 2018). Cyclodextrin-based nanosponges exhibited a superior inclusion
 89 capability than parent cyclodextrins. Indeed, they were able to incorporate many types of molecules,
 90 such as small molecules, macromolecules and gases (Trotta et al., 2014, Swaminathan et al., 2010a,
 91 Cavalli et al., 2010). The loaded molecules are generally delivered with a slow and prolonged
 92 release profile, according to the nanoparticle network structure. Indeed, the release can be
 93 influenced by the cross-linking ratio and the nature of the polymer mesh to obtain a sustained and
 94 controlled delivery (Swaminathan et al., 2010b, Torne, Ansari, Vavia, Trotta & Cavalli, 2010, Daga
 95 et al., 2016, Gigliotti et al., 2017). The incorporation and storage capability is strongly affected by
 96 the polymer nanostructure, as well as the presence of many cyclodextrin cavities able to cooperate.
 97 To load hydrophilic or charged molecules, modifications of nanosponges were devised.
 98 Interestingly, the introduction of charged groups either in the nanostructure or on the surface of
 99 nanosponges enabled the production of further interaction sites for loading dissociable drugs
 100 (Lembo et al., 2013, Bastiancich et al., 2014). Based on these premises and to expand the possibility
 101 of controlling the release, another cyclic oligosaccharide. i.e. a tetraglucose, was selected as a
 102 building block of new nanosponges, in place of cyclodextrins (Wei, et al. 2015). Intriguingly, cyclic
 103 nigerosyl-1-6-nigerose, (CNN) is a non-reducing cyclic tetrasaccharide with a unique structure
 104 consisting of four D-glucopyranosyl molecules connected by alternate α -(1-3) and α -(1-6)
 105 glycosidic bonds (Fig. 1A). CNN differs from cyclodextrin not only due to its number of glucose
 106 units (4) and type of glucosidic bond, but also due to the arrangement of the hydroxyl groups.
 107 Namely, two of them are oriented toward the inner cavity of CNN, making it quite polar.
 108 This cyclic tetrasaccharide is obtained from hydrolysed starch by the action of a mixture of
 109 enzymes (Nishimoto et al., 2002, Aga et al., 2003). Cyclotetraglucose occurs naturally in sake lees
 110 (i.e., the sediment that forms during rice wine production), in sake itself and in food-grade starch
 111 (e.g., tapioca starch, cornstarch) (Watanabe et al., 2004). The industrial manufacturing production
 112 is done by Hayashibara (Japan). This tetraglucose is a white crystalline powder which is safe and
 113 stable to temperature, while at alkaline pH values a slow degradation was observed (Weissenfeld,
 114 2005). The capacity to interact as such with various molecules was studied. The complexation of
 115 some aromatic compounds with CNN was investigated, showing the formation of guest-CN
 116 complexes with vanillin, cinnamaldehyde and eugenol (Ishikawa, Kuwano, Chaen & Matsumoto,

117 2009). Moreover, CNN have been used for powdering tocopherol, vitamin D and EPA (Oku et al.,
118 2007).

119 This work aims at exploiting the CNN unit as a monomer for the synthesis of new cross-linked
120 polymers, called CNN-nanosponges (CNN-NS). To obtain the CNN-based polymer, a synthetic
121 protocol was tuned, selecting pyromellitic dianhydride (PMDA) as a cross-linker (Figure 1B).

122 Firstly, three types of CNN-NS will be considered varying the molar ratio between CNN and
123 pyromellitic dianhydride (i.e. 1:2, 1:4, 1:6 ratios, respectively) to optimise the nanostructure for
124 drug delivery. Their physico-chemical characterisation will be studied and the drug loading
125 capability will be investigated using doxorubicin as the model molecule. Finally, the *in vitro*
126 biological behaviour of doxorubicin-loaded CNN-NS will be evaluated.

127

2. Material and methods

2.1 Materials

Cyclic nigerosyl-1-6-nigerose (CNN) was received as a kind gift from Hayashibara (Japan). Doxorubicin hydrochloride and pyromellitic dianhydride (PMDA) were purchased from Sigma. Solvents and reagents, unless otherwise indicated, were analytical-grade commercial products, used as received.

2.2 CNN-nanosponges synthesis

The quantities of chemicals used for the synthesis of the three NS are listed in table 1. In summary, 4.886 g of CNN, desiccated in an oven at 100°C up to constant weight, were solubilised in 20 mL of dimethyl sulfoxide (DMSO) at room temperature. Subsequently, 5 mL of triethylamine (Et₃N) and, after a few minutes, the correct amount of pyromellitic dianhydride were added. The solution was vigorously stirred until gelation point was reached. The obtained monolithic block was then crushed, recovered by vacuum filtration, washed with an excess of deionised water and rinsed with acetone. After drying at room temperature, the powder was collected and further purified through Soxhlet extraction for 24 h with acetone. The scheme of the CNN-NS synthesis reaction is shown in figure 1B.

Table 1. Quantities of chemicals used in the synthesis of the three types of CNN-NS

	DMSO		CNN		Et ₃ N		PMDA		PMDA/CNN
	(mL)	(mmol)	(g)	(mmol)	(mL)	(mmol)	(g)	(mmol)	molar ratio
CNN-NS (1:2)	20.0	281.6	4.886	7.534	5.0	35.9	3.286	15.067	2
CNN-NS (1:4)	20.0	281.6	4.886	7.534	5.0	35.9	6.573	30.134	4
CNN-NS (1:6)	20.0	281.6	4.886	7.534	5.0	35.9	9.859	45.202	6

153 2.3 Swelling degree evaluation

154 Dry CNN-NS specimens of known weights were immersed in buffer solutions with different pH
155 values at room temperature (i.e. pH 2.0, pH 4.0, pH 6.0 and pH 7.4 disodium hydrogen
156 phosphate/phosphoric acid buffer). At pre-determined time intervals, the swollen nanosponges were
157 removed from the buffer, blotted with filter paper to absorb the excess surface solution and
158 immediately weighed. The procedure was repeated until there was no further weight increase. The
159 swelling degree (SD) was then calculated as follows:

$$SD = \frac{(W_t - W_d)}{W_d}$$

160 where W_t is the weight of the swollen nanosponges and W_d is the weight of the nanosponges in the
161 dry state. All experiments were performed in triplicate.

162

163 2.4 X-ray powder diffraction (XRPD) analysis

164 To characterise the nanosponges, we carried out a detailed XRPD analysis using a Siemens D5000
165 diffractometer (Cu K α 1, Bragg-Brentano geometry, sequential collection between 2.5° and 60° 2 θ).
166 The diffraction patterns were analysed using Fityk software (Wojdyr, 2010).

167

168 2.5 DSC analysis

169 Differential Scanning Calorimetry (DSC) was carried out by means of a Perkin Elmer DSC/7
170 differential scanning calorimeter (Perkin-Elmer, CT-USA) equipped with a TAC 7 /DX instrument
171 controller. The instrument was calibrated with indium for melting point and heat of fusion. A
172 heating rate of 10°C/min was employed in the 25-250°C temperature range. Standard aluminium
173 sample pans (Perkin-Elmer) were used; an empty pan was used as a reference standard. Analyses
174 were performed in triplicate on 3 mg samples under nitrogen purge.

175

176 2.6 Preparation of doxorubicin-loaded CNN-nanosponges (1:4)

177 An aqueous suspension of the CNN-NS(1:4) at the concentration of 10 mg/ml was prepared. A pre-
178 homogenisation was carried out for 10 min of Ultraturrax (Ika, Germany) at 24,000 rpm. The
179 aqueous suspension was then transferred into a high pressure homogeniser (HPH) (Emulsiflex C5,
180 Avestin, USA) and subjected to homogenisation. The protocol tuned consists of 5 cycles at 5,000
181 psi for 5 min, 12 cycles at 7,000 for 90 min. The obtained aqueous nanosuspension was purified by
182 dialysis and stored at 4°C. An aqueous solution of doxorubicine (2 mg/ml) was incubated at room
183 temperature with the CNN nanosuspension under mild stirring for 12 hours. Subsequently, a

184 dialysis step was performed to eliminate the unloaded doxorubicin. For cell experiments the
185 nanosuspension was prepared in NaCl 0.9% w/v aqueous solution.

186

187 *2.7 Quantitative determination of doxorubicin*

188 The quantitative determination of doxorubicin was carried out by a HPLC system consisting of a
189 pump (LC-9A PUMP C, Shimadzu, Japan) equipped with a fluorescence detector (Chrompack,
190 Japan). Analyses were performed using an Agilent TC C₁₈ column (250 mm × 4.6 mm, 5 µm). The
191 mobile phase was a mixture of 0.01 M KH₂PO₄ (pH 1.4), acetonitrile and methanol (65:25:10
192 v/v/v), degassed and pumped through the column at a flow rate of 1 ml/min. The column effluent
193 was monitored at excitation and emission wavelengths of 480 and 560 nm, respectively. The
194 external standard method was used to calculate the drug concentration. For this purpose, 1 mg of
195 doxorubicin was weighed, placed in a volumetric flask, and dissolved in water to obtain a stock
196 standard solution. This solution was then diluted using the mobile phase, providing a series of
197 calibration solutions, subsequently injected into the HPLC system. The linear calibration curve was
198 obtained over the concentration range of 0.025–2.5 µg/ml with a regression coefficient of 0.999.

199

200 *2.8 Determination of doxorubicin encapsulation efficiency*

201 A 10 mg/mL aqueous suspension of the freeze-dried loaded nanosponges was prepared using
202 filtered water and doxorubicin extracted by sonication for 5 minutes at room temperature. After
203 centrifugation, the drug concentration in the supernatant was determined by the HPLC method
204 previously described. Encapsulation efficiency and drug loading were then calculated using the
205 calibration curve.

206

207 *2.9 Size, polydispersity index and zeta potential values*

208 CNN-NS sizes and polydispersity indices were measured by dynamic light scattering using a 90
209 Plus particle sizer (Brookhaven Instruments Corporation, USA) equipped with MAS OPTION
210 particle sizing software. The measurements were made at a fixed angle of 90° for all samples,
211 which were suitably diluted with filtered and distilled water for every measurement. The zeta
212 potential measurements were also taken using an additional electrode in the same instrument. For
213 zeta potential determination, samples of the three formulations were diluted with 0.1 mM KCl and
214 placed in the electrophoretic cell, where an electric field of about 15 V/cm was applied.

215

216 *2.10 Solid state NMR analysis*

217 Solid state NMR spectra were acquired using a Jeol ECZR 600 instrument, operating at 600.17 and
218 150.91 MHz for ^1H and ^{13}C nuclei, respectively. Powder samples were packed into cylindrical
219 zirconia rotors with a 3.2 mm OD and a 60 μL volume. A certain amount of sample was collected
220 from each batch and used without further preparations to fill the rotor. ^{13}C CPMAS spectra were
221 acquired at a spinning rate of 20 kHz using a ramp cross-polarisation pulse sequence with a contact
222 time of 3.5 ms, a 90° ^1H pulse of 2.189 μs , (optimised) recycle delays of 3.5 s (doxorubicin), 1.9s
223 (blank CNN-NS (1:4)) and 0.4s (doxorubicin-loaded CNN-NS(1:4)) and a number of scans
224 included between 2000 and 10000, depending on the sample. For every spectrum a two pulse phase
225 modulation (TPPM) decoupling scheme was used, with a radiofrequency field of 108.5 kHz. The
226 chemical shift scale was calibrated through the methylenic signal of external standard glycine (at
227 43.7 ppm).

228

229 *2.11 Morphology analysis*

230 Transmission electron microscopy (TEM) was used to evaluate particle shape and morphology. A
231 Philips CM 10 transmission electron microscope was used and the particle size was measured using
232 the NIH image software. The nanosponge suspensions were sprayed on Formwar-coated copper
233 grid and air-dried before observation.

234 Scanning electron microscopy (SEM) was used to evaluate the morphology of the CNN-NS
235 formulations using a JEOL JSM IT300LV (EHT 20 kV, working distance 10 mm). The
236 nanosponges were positioned on a conductive sample holder and sputtered with graphite to assure
237 the conductivity of the sample.

238

239 *2.12 ATR FTIR analysis*

240 ATR FTIR analysis was applied to doxorubicin-loaded CNN-NS, free doxorubicin and plain CNN-
241 NS using a Perkin Elmer 2000 instrument. The spectra were recorded between 400 and 4000 cm^{-1} .

242

243 *2.13 Stability evaluation of CNN-nanosponges*

244 The physical stability of CNN-NS was evaluated over time, determining size, surface charge and
245 doxorubicin loading, as previously described. This thermal stability of CNN-NS can allow for
246 sterilisation by autoclaving for cell experiments.

247

248 *2.14 In vitro release of doxorubicin from CNN-nanosponges (1:4)*

249 The *in vitro* release was carried out using multi-compartment rotating cells with a dialysis
250 membrane (Sartorius, cut off 12,000 Da). The donor phase consisted of nanosponge formulation
251 containing a fixed amount of doxorubicin in phosphate buffer at pH 7.4 (1 ml). The receiving phase
252 consisted of phosphate buffer, pH 7.4 or pH 5.5 added with 0.5% w/v sodium lauryl sulphate (1 ml)
253 to maintain proper sink conditions. The receiving phase was completely withdrawn and replaced
254 with fresh medium after fixed time intervals, suitably diluted and analysed using the HPLC method
255 described above.

256

257 2.15 Biological assays

258

259 2.15.1 Haemolytic activity of CNN-nanosponges

260 CNN-NS (10 mg/mL) were incubated at 37°C for 90 minutes with 1 ml of diluted blood. Freshly
261 prepared PBS (pH 7.4) was used for all dilution purposes. After incubation, blood-containing
262 suspensions were centrifuged at 2,000 rpm for 10 min to separate the plasma. The amount of
263 haemoglobin released due to haemolysis was measured spectrophotometrically at 543 nm (Du 730,
264 Beckman). The haemolytic activity was calculated with reference to blank and complete
265 haemolysed samples (induced by the addition of ammonium sulphate 20% w/v). Optical
266 microscopy was also used to see if there were any abnormalities in the blood cells after incubation.
267 The observations were made with reference to the blank diluted blood. No changes in red blood cell
268 morphology were detected.

269

270 2.15.2 *In vitro* cytotoxicity determination

271 The ovarian cell line, A2780, was obtained from the American Type Culture Collection (Rockville,
272 MD, USA). The cells were grown as a monolayer culture in RPMI 1640 medium supplemented
273 with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/l L-glutamine and
274 penicillin/streptomycin (100 units/ml), at 37°C in 5% CO₂ humidified atmosphere.

275

276 2.15.3 Colony-forming assay

277 Cells (1,000 per well) were seeded into six-well plates and treated with the compounds. The
278 medium was changed after 72 h and cells were cultured for an additional 10 days without any drug.
279 Subsequently, the cells were fixed and stained with a solution of 80% crystal violet (Sigma-Aldrich)
280 and 20% methanol for 30 min. The cells were then perfectly washed and 30% acetic acid was added

281 to induce a complete dissolution of the crystal violet. Absorbance was recorded at 595 nm by a 96-
282 well-plate ELISA reader. Five different experiments were performed.

283

284 *2.15.4 In vitro uptake study*

285 5×10^4 cell/well of A2780 cells were seeded in 24-well plates in 1 ml of culture medium and treated
286 with 10^{-7} M free Doxorubicin or CNN Doxorubicin. 48 h (T48) later the cells cultured on the
287 coverslips were rinsed three times with cold PBS and fixed with 75% ethanol for 30 min at 4°C and
288 the coverslips were inverted and mounted on glass slides. The nuclei were stained with 4',6'-
289 diamidino-2-phenylindole (DAPI 1 mg·mL⁻¹; Sigma-Aldrich, Saint Louis, MO, USA). The slides
290 were analysed by confocal laser scanning microscopy (CLSM) using a Leica DMIRE2 confocal
291 fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with Leica
292 Confocal Software v. 2.61.

293

294 *2.16. Statistical analysis*

295 Data are shown as mean $\pm$ SEM. Statistical analyses were performed with GraphPad Prism 3.0
296 software (La Jolla, CA, USA) using one-way ANOVA and Dunnett's test. Values of $P < 0.05$ were
297 considered statistically significant.

298

299

300   **3. Results and Discussion**

301   One strategy to improve the therapeutic effectiveness and to reduce the side effects of anticancer
302   drugs on healthy cells is to improve local drug concentrations at the disease tissues. Biocompatible
303   nanoparticles containing drugs can provide a site-specific delivery of anticancer drugs in a
304   controlled and sustained manner improving the safety profile (Brigger, Dubernet & Couvreur,
305   2002). Nanoscaled delivery systems can carry loaded drugs to the tumour site through the
306   bloodstream, taking advantage of the enhanced permeability and retention (EPR) effect, due to the
307   defective vascular architecture of the tumour (Fang, Nakamura & Maeda, 2011, Maeda, Nakamura
308   & Fang, 2013). Stimuli-responsive polymer nanoparticles can allow more precise and controlled
309   release behaviour (Cheng, Meng, Deng, Klok & Zhong, 2013). Based upon these concepts, a new
310   nanodelivery system has been developed, using a biocompatible tetraglucose, i.e. cyclic nigerosyl-
311   1-6-nigerose (CNN), as an innovative building block. Solid cross-linked polymer nanoparticles,
312   named cyclic nigerosyl-1-6-nigerose based nanosponges (CNN-NS) were prepared, by the reaction
313   of CNN with pyromellitic dianhydride, to obtain pH responsiveness. Figure 1B reports the schematic
314   representation of the CNN-NS synthesis reaction.

315

Figure 1. A) Chemical structure of cyclotetraglucose (CNN) B) Scheme of the CNN-NS synthesis reaction C) ATR-FTIR spectra of CNN and CNN-NS D) Swelling capacity of CNN-NS (1:2) as a function of pH values (pH 2 and 7.4).

PMDA was selected, among several cross-linkers, for its high reactivity towards hydroxyl groups, which allows fast reactions with high yields and because of the interactions that carboxylic groups of pyromellitic bridges may establish with polar moieties of drugs (i.e. -COOH, -NH₂, -OH groups). These interactions might contribute to high encapsulation efficiency and slow release kinetics. Moreover, the high content of carboxylic groups, introduced by PMDA, confers a pH-sensitive character to CNN-NS, which may be profitably exploited to modulate the release of loaded drugs. The presence of the cross-linked network was confirmed by FTIR analysis. Indeed, the absorption band typical of carboxylate groups appeared in the FTIR-ATR spectrum of CNN-NS at 1580 cm⁻¹, along with other signals deriving from pyromellitic units (i.e. C=O stretching in

330 carboxylic groups at 1720 cm^{-1} and C-O stretching in ester groups at 1240 cm^{-1} , approximately) was
331 not detected in the spectrum of pristine CNN (figure 1C).

332 The synthetic process is one-step, cost-effective and its repeatability and scalability have been
333 confirmed. After synthesis, the solid CNN-NS underwent to the HPH step to decrease the
334 nanoparticle dimensions and to produce smaller sizes and more uniform distribution. Indeed, the
335 CNN-NS sizes were reduced reaching sizes lower than 400 nm.

336 Here, the optimisation and *in vitro* characterisation of this new tetraglucose-based nanodelivery
337 system are reported. Firstly, the effect of the three different CNN cross-linker ratios (i.e. 1:2, 1:4
338 and 1:6) on the nanostructure and physico-chemical properties was investigated to characterise the
339 three products. All the obtained CNN-NS are amorphous solids, although CNN as such is markedly
340 crystalline. Figure 2 shows the XRPD diagrams of the three types of CNN-NS compared with that
341 of nigerose.

342

343

344

345 **Figure 2.** X-ray powder diffraction patterns of the CNN-NS obtained by reacting the CNN with
346 variable concentrations of pyromellitic dianhydride (1:2, 1:4, 1:6 respectively) compared with the
347 XRPD of the nigerose (bottom).

348

349 Despite the high degree of crystallinity displayed by nigerose, CNN-NS highlight their amorphous
350 behaviour, shown by the broad bump in the range 10-40 °2θ.

351 The amorphous nature was confirmed by DSC analyses. No peaks correlated to thermal changes
352 were observed until 250°C (data not shown).

353 CNN-NS were easily able to absorb water and their swelling capacity was investigated as a function
354 of the pH value of the external environment.

355 The three types of CNN-NS showed a marked swelling capacity related to the cross-linking degree,
356 which remarkably depends on the pH value (Table 2).

357 Interestingly, CNN-NS were pH sensitive due to the presence of dissociable carboxylic groups in
358 the polymer matrices. In particular, the swelling degree of the three types of nanosponges linearly
359 increased from pH 2.0 to pH 7.4.

360 Table 2 reports the swelling degree of the two nanosponges as a function of pH values of the
361 external medium.

362

363 **Table 2.** Swelling degree of CNN-NS as a function of pH values of the external medium.

Sample	Swelling degree (%)			
	pH 2.0	pH 4.0	pH 6.0	pH 7.4
CNN-NS (1:2)	562	680	765	820
CNN-NS (1:4)	124	215	307	415
CNN-NS (1:6)	88	206	248	392

364

365 It can be seen that CNN-NS (1:2) demonstrated a higher water uptake capability at all the pH values
366 than those of the other two CNN-NS with higher degrees of cross-linking (Figure 1D).

367 Indeed, the lower the cross-linking degree, the higher the observed swelling. Interestingly, CNN-NS
368 can be considered a quasi super-absorbent material, that was able to retain more than 800 wt% of
369 water at room temperature and pH 7.4.

In this work, doxorubicin was selected as a model drug. Doxorubicin is one of the most common anticancer drug, but it suffers of severe side effects and multidrug resistance. Therefore a nanoformulation to improve its pharmacokinetics and biodistribution would be valuable. Preliminary explorative experiments proved that CNN-NS (1:4) were the most suitable for loading and storing doxorubicin. As a consequence, this type of CNN-NS was selected for further investigation and characterization with the drug.

Table 3 reports the physico-chemical characterization of doxorubicin-loaded and unloaded CNN-NS (1:4).

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379
380
381
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383
384

Table 3. Physico-chemical characterization of blank and doxorubicin-loaded CNN-NS

386
387

	CNN-NS (1:4) (unloaded)	Doxorubicin-loaded CNN-NS (1:4)
Average diameter $\pm$ SD (nm)	355.6 ± 27.3	360.8 ± 15.6
PDI	0.20 ± 0.02	0.19 ± 0.03
PZ $\pm$ SD (mV)	-30.2 ± 4.55	-29.2 ± 5.29
Loading capacity (%)	-	15.5
Encapsulation efficiency (%)	-	95.1

388

Doxorubicin encapsulation in CNN-NS did not require high-energy processes or solvent use preserving the drug from potential degradation. Interestingly, it is incorporated by incubation at room temperature under mild stirring. Doxorubicin is a hydrophilic drug with a good aqueous solubility (10 mg/ml). The hydrophilicity of the polymer mesh and probably the polarity of the CNN cavity provide drug encapsulation with high efficiency. The polymer network should play the

key role in the drug incorporation. Indeed, the CNN monomer possesses only a shallow saucer-like shape with a small concave at the center, confirmed by X-ray crystallographic analysis (Bradbrook et al., 2000, Yang et al., 2012). Considering this CNN architecture, the doxorubicin might be mainly entrapped in the hydrophilic nanochannels of the polymer network. The drug loading was 15.5% for doxorubicin-loaded CNN-NS (1:4), with an encapsulation efficiency of about 95%. Figure 3 shows the SEM (A) and TEM (B) images of CNN-NS before and after HPH step respectively.

Figure 3. A) SEM image of CNN-NS before HPH step and B) TEM image of the CNN-NS after HPH step (TEM scale bar 300 nm).

Figure 3A shows the irregular morphology and large particle size of NS prior to HPH treatment. As reported in the literature (Trotta, 2011), dextrin-based NS have generally rather compact structures. Also in the case of CNN-NS, BET analysis revealed surface area values below 2 m²/g (data not shown). Cyclodextrin- and CNN-NS have nanometric and sub-nanometric intrinsic porosity, deriving from the inner cavities of dextrin monomers and from the interstitial volumes among dextrin monomers. Such molecular-level porosity cannot be discerned by means of scanning electron microscopy. TEM image (Fig. 3B) shows the CNN-NS spherical shape and confirms the nanoscaled sizes, reached after the homogenization process.

When loaded with doxorubicin, CNN-NS lose their frankly amorphous behaviour, increasing their low range ordering. Indeed, the new order does not tally with the structure of crystalline doxorubicin, as shown in Figure 4A. The presence of a few broad diffraction peaks in the diffraction pattern of the loaded CNN-NS demonstrates the formation of a brand new, poorly

418 ordered phase; that ordering is presumably due to the inkling ordering of the doxorubicin inside the
 419 nanosponge.

420
 421

422 **Figure 4.** A) CNN-NS (1:4) loading with doxorubicin leads to a deep modification of the
 423 nanosponge diffraction pattern (doxorubicin-loaded CNN-NS (1:4) in figure) B) Experimental data
 424 (dots) and calculated function (red curve) of XRPD of the doxorubicin-loaded CNN-NS. Broad
 425 yellow curves represent the decomposition of the XRPD pattern, while the blue one corresponds to
 426 the amorphous background.

428

429 **Table 4:** Position, height, area and full width at half maximum of the three diffraction peaks of the
 430 CNN 1:4 loaded with doxorubicin

431

#	Center (°2θ)	Height (cps)	Area (cps)	FWHM (°2θ)
1	21.3426	37.5097	478.982	11.9962
2	36.6454	23.6005	60.563	2.41077
3	51.4022	16.0661	48.7831	2.85251

432
 433
 434
 435

436 The diffraction data of the CNN 1:4 nanosponges with doxorubicin were decomposed using the
437 Fityk software (Wojdyr, 2010). The decomposition of the XRPD pattern shows at least three curves
438 representing the poor ordering of the system.

439 Due to the broad behaviour of the peaks, a Gaussian profile fitting was selected. The pattern shows
440 a bump (blue line in Figure 4B) ascribable to the amorphous phase and three broad peaks (yellow)
441 possibly related to the weak ordering of the doxorubicin in the loaded nanosponge.

442 DSC analysis of doxorubicin-loaded CNN-NS showed the drug incorporation in the nanosponge
443 matrix (Figure 5A). Indeed, the endothermic peak related to the melting of doxorubicin at about
444 220°C is missing in the thermograms of doxorubicin-loaded CNN-NS, indicating that the drug is
445 molecularly dispersed in the polymer matrix.

446 The cross-linked structure of CNN-NS was confirmed by ¹³C solid state NMR analysis. Signals
447 deriving from carbonyl groups in ester/carboxylic moieties and aromatic carbon atoms belonging to
448 PMDA units appear at approximately 170 and 130 ppm, respectively (figure 5B).

449 In addition, solid state NMR confirmed the interaction of the drug with CNN-NS.

Figure 5. A) DSC thermograms of CNN-NS (1:4), free doxorubicin and doxorubicin-loaded CNN-NS. **B)** Solid NMR profiles of CNN-NS (1:4), free doxorubicin and CNN-NS. **C)** Comparison between ^{13}C CPMAS spectra of doxorubicin, blank nanosponge and doxorubicin-loaded CNN-NS in the 0-220 ppm range. Arrows and chemical shift values facilitate the identification of significant peaks.

Figure 5b shows the ^{13}C CPMAS SSNMR spectra of doxorubicin, blank CNN-NS (1:4) and doxorubicin-loaded CNN-NS (1:4). Figure 5c shows the comparison between the spectra of the three samples. In the spectrum of the loaded sample (doxorubicin-loaded CNN-NS, red) several signals, different from the characteristic peaks of the CNN-NS (blank nanosponges, blue), are observed and indicated with arrows. These signals are compliant with the typical chemical shifts of the ^{13}C atoms of doxorubicin (also shown in the figure) and thus evidence the presence of doxorubicin inside the nanosponges.

Then, the *in vitro* doxorubicin release kinetics from CNN-NS was evaluated at pH 5.5 and at pH 7.4 (Figure 6A).

Figure 6. A) *In vitro* release kinetics of doxorubicin from CNN-NS (1:4) as a function of pH; **B)** *In vitro* release kinetics of doxorubicin from CNN-NS (1:4) at pH = 7.4

In vitro release profiles showed a very slow and constant release of the drug over time depending on the pH value of the receiving phase. Interestingly, no burst effect was observed at both pH values. After 24 hours only 1.3% of doxorubicin was released from CNN-NS at pH 7.4, while the percentage of doxorubicin released at pH 5.5 reached 10%. The slow and pH-sensitive release profile may be related to the chemical structure of CNN-NS. The main role is played by the polymer matrix, which comprises free carboxylic groups in the polymer network. The carboxylic groups, belonging from the pyromellitic dianhydride used as cross-linker, are only partially dissociated at pH 5.5 resulting in lower interactions and favouring the drug release. On the other hand, the cavity of CNN monomer is not suitable for doxorubicin complexation being a very small pocket, as previously reported. Considering the β -cyclodextrin-based NS obtained by reacting β -CD

483 with pyromellitic dianhydride, a doxorubicin release of about 10% was reached after 6 hours at pH
484 7.4 (data not shown). This different behaviour might be related to the narrow polymer meshes
485 present in CNN-NS. Indeed, the extremely slow and prolonged release behaviour from CNN-NS
486 compared to other doxorubicin nanosponge formulations at pH 7.4 (Cavalli, Trotta & Tumiatti,
487 2006) might represent an advantage to limit the drug blood exposure and the toxicity of
488 doxorubicin.

489 We speculate that doxorubicin may be released in a controlled manner following Fickian kinetics
490 due to drug diffusion through the polymeric matrix, which underwent degradation with a very slow
491 kinetics due to hydrolysis. Indeed, the absence of any burst effect could indicate that doxorubicin is
492 not adsorbed on the nanosponge surface and CNN-NS are slowly degraded in the physiological
493 environment.

494 The release mechanism was further evaluated. The doxorubicin *in vitro* release from drug-loaded
495 CNN-NS was fitted to four distinct models to determine which one exhibited the highest correlation
496 with the experimental results. For each model, the rate constant and correlation values were
497 obtained by applying a linear regression fit. The zero-order kinetic model demonstrated the higher
498 correlation, showing R^2 values of 0.9969 and 0.9985 for doxorubicin release at pH 7.4 and pH 5.5,
499 respectively.

500 The *in vitro* release kinetics of doxorubicin from CNN-NS at pH 7.4 was followed up to 21 days
501 (Figure 6B).

502 Intriguingly, the results indicate that at physiological pH value, CNN-NS provide a slow drug
503 diffusion and controlled release over a lengthy period. Indeed, after 14 days at pH 7.4 only about
504 20% of the drug was released and after 21 days this reached about 32.5%. This behaviour could be
505 exploited to obtain a constant drug concentration in tumour tissue in loco-regional treatment (Cheng
506 et al., 2017, Cinar et al., 2017). Interestingly, the tumour environment pH is more acid than that of
507 normal tissue (Manchun, Dass & Sriamornsak, 2012).

508 In order to verify the absence of nanosponge activity, cells were treated with plain CNN-NS at
509 dilutions corresponding to that drug-loaded CNN-NS. In all the concentrations tested, the results
510 overlapped with those obtained in controls, with no significant inhibition of cell proliferation, thus
511 excluding any toxic effect of the nanocarriers.

512 Concerning CNN-NS safety, no significant hemolysis caused by CNN-NS either blank or
513 doxorubicin loaded was observed, confirming their good biocompatibility and the presence of
514 tonicity values suitable for a future potential *in vivo* administration.

515 Clonogenic survival assays were performed. The cells were seeded 6-well plates and treated with
 516 each compound. The culture medium was changed after 72 h, and the cells were cultured for an
 517 additional 10 days in the absence of the compounds. The results showed that doxorubicin or CNN
 518 doxorubicin treatments differently inhibited the ability of the A2780 cell line to form colonies. In
 519 fact, both formulations induced the maximum effect at 10^{-7} M, but doxorubicin-loaded CNN-NS
 520 were able to induce a higher inhibition of the colony formation at 10^{-8} - 10^{-9} M, determining growth
 521 inhibition around 80% and 50%, respectively. Conversely, the treatment with doxorubicin in
 522 solution produced a smaller inhibition of the ability to form colonies (50%) at 10^{-8} M, being no
 523 more active at the lower concentration.

526
 527 **Figure 7.** Effect of doxorubicin and doxorubicin-loaded CNN-NS on cell clonogenicity was tested
 528 by colony forming assay. A2780 cells (1000 per well) were seeded in six-well plates and treated
 529 with each drug at the indicated concentrations for 72 h. The medium was then changed and cells
 530 were cultured for additional 10 days and subsequently fixed and stained with crystal violet. Data
 531 shown are means $\pm$ SEM ($n = 5$). ** $P < 0.01$, * $P < 0.05$ significantly different from the same
 532 concentration of doxorubicin.

534 Indeed, the IC_{50} was $0.9 \pm 0.2 \times 10^{-9} M$ and $8.5 \pm 1 \times 10^{-9} M$, respectively for doxorubicin-loaded
 535 CNN-NS and for free doxorubicin. Therefore, the cytotoxicity of doxorubicin loaded in
 536 nanosponges was enhanced of about 8 fold than free drug on A2780 cell lines.
 537 Cell uptake is promoted by nanocarriers as previously shown with various nanoformulations
 538 (Miglietta, Cavalli, Bocca, Gabriel & Gasco, 2000, Ernsting, Murakami, Roy, & Li, 2013, Wang et
 539 al., 2016). The size, surface charge and the components of CNN-NS might favored the cell
 540 internalization. Indeed, *in vitro* uptake results demonstrated that fluorescence was localized in the
 541 cytoplasm around the nucleus to a greater extent in the cells treated with doxorubicin-loaded CNN-
 542 NS, indicating that CNN-NS had been internalised in a greater extent into the cells.
 543

544
 545 **Figure 8.** Cell uptake was evaluated by fluorescence microscopy analysis using different doses of
 546 doxorubicin-loaded CNN-NS (DOXO-CNN) o free DOX on A2780 cells for 48 h.
 547

548 Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of
 549 a single cell to grow into a colony. Only a fraction of seeded cells retains the capacity to produce
 550 colonies. After plating at very low density (800 cells for wells), cells are treated with the
 551 compounds for 72h, thus they were washed with the cell medium, to remove drugs, and were then
 552 allowed to grow over an extended period of time (10 days). Doxorubicin-loaded CNN-NS may act
 553 as intracellular drug reservoir slowly releasing the free drug into the cellular cytoplasm, enhancing
 554 its therapeutic efficacy. Doxorubicin encapsulation in CNN-NS might increase its concentration at

the tumor site, thereby decreasing the frequent dose administered and subsequently reducing the side effects. In this *in vitro* assay, free DOX is also able to inhibit the colony formation, especially at the highest concentration tested (10^{-7} M), considering the static conditions of the experimental set-up. We can hypothesize a different behaviour *in vivo*. Various factors controlled the pharmacokinetics, biodistribution and intratumoral penetration of nanoparticles after their *in vivo* administration (Ernsting et al., 2013). A crucial role is played by the EPR effect, that allows the nanoparticles to escape from the vessels and enter in the tumor cells.

562

563 **4. Conclusions**

A new polymer-based nanomaterial was obtained, exploiting a tetraglucose, i.e. CNN, as a monomer in the synthetic reaction. The cross-linking reaction with pyromellitic dianhydride formed solid nanoparticles, named nanosponges. This new nanomaterial is biocompatible and is able to swell in response to the pH value. CNN-NS can be formulated as nanoscale particles with spherical shape suitable for drug delivery. Doxorubicin was incorporated in a good extent and released with a very slow and constant kinetics. Interestingly, the environment pH plays a role in controlling the release profile of the drug.

Based on the results, doxorubicin-loaded CNN-NS might act as a nanomedicine tool for tumor local treatment with a favorable toxicology profile.

573

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577

578 **Author contribution**

FC synthesized the CNN-nanosponges, MA and MT formulated and *in vitro* characterized the nanoformulations, LG and CD performed cell culture experiments, LP and DA performed XRPD analyses, FT and RC designed the experiments and gave the intellectual rationale to the work, TN and TH samples gift and chemical information on cyclic nigerosyl-1-6-nigerose.

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