Electronic supplementary information

1.1 Techniques for the determination of chitosan degree of deacetylation – experimental part

Absorption or emission spectroscopy

Colloid titration was performed in a 0.02M acetate buffer/0.1M NaCl, pH 4.5 solution, as described by Berth et al. [1]. The titration was carried out directly in a 10mm cuvette on a Perkin Elmer Lambda 850 spectrometer. 2ml of 5- 15μg/ml chitosan solution was mixed with 20μl of 0.03% toluidine blue O solution (a cationic metachromatic indicator). The solution was then added in steps of 10μl of a PPS solution 1mM in sulfonate groups, while recording the difference of the absorbance values at 635 and 600nm. The end points of the titration were calculated by fitting the data with the Boltmann equation and using the inflection point (OriginPro 8).

The **ninhydrin test** was carried out following the method of Prochazkova *et al*. [2]. Chitosan was dissolved in 2% acetic acid solution at a concentration of 0.1mg/ml. 500μl of chitosan solution were diluted to 1ml with distilled water and 1ml of 2% ninhydrin reagent solution was then added in the chitosan solution. The tubes were placed in a boiling water bath for 10min. After the tubes were cooled in a cold water bath, the solutions were diluted with 5ml of 50%$^{v}/_{v}$ ethanol/water solution. They were then vigorously stirred on a vortex mixer for 15sec to oxidize the excess of hydrindantin. The reaction of ninhydrin with a primary amino group formed a coloured reaction product, recording the absorbance value at 580nm.

In the **fluorescamine assay** primary amine groups on D-glucosamine residues of chitosan were determined as described in the method by Corsi *et al*. [3]. Chitosan was dissolved in 50mM acetic acid solution, which was then adjusted to pH 5.5. 10μl of sample were diluted in 140μl of boric acid-NaOH (100mM) pH 8 in a 96-well plate. 100μl of 0.01%$^{w}/_{v}$ fluorescamine solution (in acetone) was added into each well, mixed and incubated for 10min at room temperature. The fluorescence intensity was measured at an excitation wavelength of 390nm and an emission wavelength of 475nm.

It is useful to remind that the absorption or emission intensities are related to the concentration of the polymer expressed in g/L ($C_{chitosan}$) and to the weight fraction of glucosamine units W_{gluNH_2}, which then allows the calculation of their molar fraction x_{gluNH_2} as described hereafter (specifically for the case of absorbance):

$$A_x = \varepsilon_x lM = \varepsilon_x l \frac{C_{chitosan} W_{gluNH_2}}{MW_{gluNH_2}} \text{ where } W_{NH_2} = \frac{MW_{gluNH_2} x_{gluNH_2}}{MW_{gluNH_2} x_{gluNH_2} + MW_{glu\_amide} x_{glu\_amide}}$$

where x_{gluNH_2} and $x_{glu-amide}$ are respectively the molar fractions of glucosamine and glucosamide repeating units and $x_{gluNH_2} + x_{glu\_amide} = 1$

IR spectroscopy

The typical diagnostic bands for chitosan are located at 3500-3000 (ν OH and NH_2), 2876 (ν CH (not CH_3)), 1655 (amide I), 1560 (amide II), 1375 (symm. δ CH_3), 1310 (δ C-N-H), 1063 and 1030 (ν C-O) cm^{-1}.

The C-H stretching absorption at 2876 cm^{-1} is not influenced by the degree of deacetylation; we have therefore used the intensity of this band, separated by other bands related to OH and NH stretching vibrations (Figure 1 SI), as a reference to normalize the peaks sensitive to the presence of acetamide residues, i.e. **(1)** amide I at 1655 cm^{-1}, **(2)** amide II at 1560 cm^{-1}, **(3)** CH_3 symmetrical bending at 1375 cm^{-1}, and **(4)** amide III, C-N-H bending at 1310 cm^{-1}. Comparing the normalized absorptions of these four bands with those of the fully acetylated chitin it is possible to calculate a degree of deacetylation as

$$\%DD = [1 - \frac{(A_{xxxx}/A_{2876})^{chitosan}}{(A_{xxxx}/A_{2876})^{chitin}}]*100$$

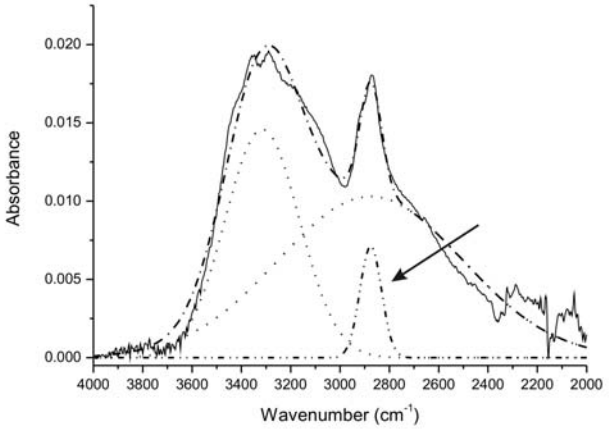

Figure 1SI. The C-H stretching band (identified by an arrow) can be easily separated from other underlying bands in the area by multi-peak fittings with Gaussian curves.

^{1}H-NMR

^{1}H-NMR (0.5 M DCl/D_2O): δ (ppm) = 1.85-1.90 (acetamide C**H$_3$**), 3.00-3.05 (C**H**-NH_2), 3.55-3.60 and 3.70-3.75 (two broad peaks comprising C**H**-$NHCOCH_3$ and all other non-anomeric protons; H3, H4, H5, H6, H6' of hexosamine residues).

The DD was calculated using the integral intensity of acetyl groups (H_{Ac}) and the average of the integral intensities of H2, H3, H4, H5, H6 and H6' protons (H_{2-6})[4] as

$$\%DD = \left[1 - \frac{H_{Ac}/3}{H_{2-6}/6}\right]x100$$

Elemental analysis

The DD was calculated from the carbon/nitrogen ratio (C/N). The C/N would vary from 5.145 to 6.861 for completely N-deacetylated chitosan ($C_6H_{11}O_4N$ repeat units, represented 100% DD) and fully N-acetylated chitin ($C_8H_{13}O_5N$ repeat units, represented 0% DD), respectively. The DD was calculated according to the following equation:

$$\% \text{ DD} = 100 - [\frac{C/N - 5.145}{6.861 - 5.145} x100]$$

1.2 Techniques for the determination of chitosan degree of deacetylation – results and discussion

The comparison between different analytical methods is of the essence for obtaining reliable degree of deacetylation (DD) data. Generally, the DD of chitosan can be determined by detecting either the primary amine groups in D-glucosamine residues (we have used fluorescamine assay, ninhydrin test and colloid titration) or the acetamide groups in N-acetyl-D-glucosamine residues (we have used IR and NMR).

Fluorescamine assay: it is a simple and rapid fluorometric method [5]; here we have used glucosamine as a standard. Our data showed a complete failure of this method in the detection of chitosan primary amines, providing concentrations markedly lower than the other methods. We speculate that this may be related to issues of steric hindrance; indeed this test works very well on low MW compounds and it has been successfully used also on short chain chitosans[6].

Ninhydrin test: this test, based on UV-Vis absorption,has been used to determine DD of chitosan in the past, in some cases providing DD values close to those of potentiometric titration measurements[7], in some others much lower than other methods[8]. Indeed it has been shown that the ninhydrin reactivity of chitosan with high DD and MW is lower than that of the monosaccharide glucosamine [2]. It was not surprising that this method has provided us an unrealistic DD of 32%,

Colloid titration: the DD was measured by titrating chitosan with negatively charged polymers, such as PSS [1] and poly(vinyl sulphate) [9], and using toluidine blue as a metachromatic indicator. The DD of chitosan calculated from colloid titration was >90%, which is in good agreement with that determined by other spectroscopic methods. It must be pointed out that chitosan oligomers less than tetrameric cannot be determined accurately by this method due to the low stability constant of the polyelectrolyte complexes between oligo-chitosan and PSS [10].

Infrared spectroscopy: different probe and internal standard bands have been suggested to determine the DD depending on the content of acetyl groups [11, 12]. Among the various probe bands used, the peak at 1655cm^{-1} (amide I) is the most popular one for determination of DD, particularly with high DD values [13, 14]. Both the OH stretching at 3450cm^{-1} [12, 14] and the CH stretching at 2877cm^{-1} [13, 15] have been commonly used as reference bands. However, the CH stretching at 2877cm^{-1} is more reliable because the water content in the sample influences the absorption band at 3450cm^{-1} [16]. In our experiments, the DD of undegraded chitosan varied from 75.13% to 87.47% depending on the bands used for calculation.

^{1}H-NMR: the analysis of proton spectra provided values largely in agreement with the results of colloidal titration, IR (above all amide I data) and elemental analysis.

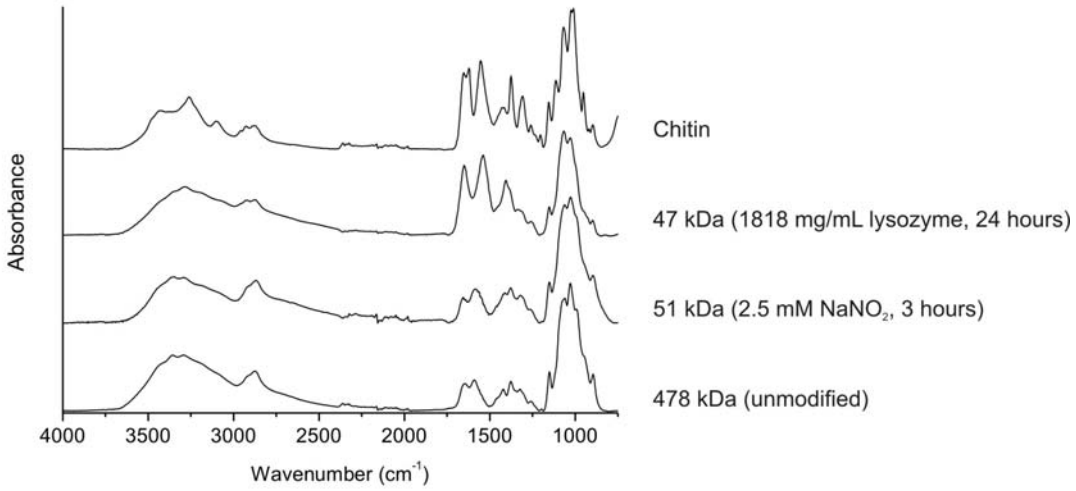

Figure 2SI. IR spectra of different chitosan samples (conditions of degradation added) and of chitin, which was used as a reference. The differences in the amide band region clearly show how, for example, the enzymatic degradation with lysozyme markedly reduced the degree of deacetylation.

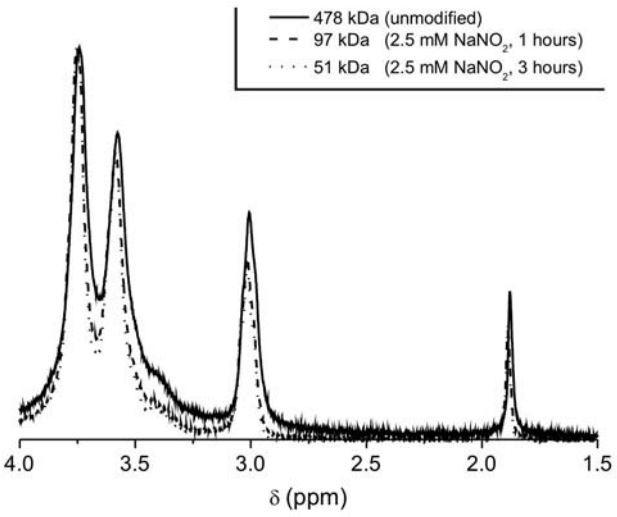

Figure 3SI. Typical ^{1}H-NMR spectra of chitosan samples.

According to the supplier's specification, the undegraded chitosan had a DD of 75-85%, which was comparable with the results of colloidal titration, IR, NMR and elemental analysis experiments (Table 1SI). Due to the simplicity and low cost, colloidal titration, IR and NMR were further used for the characterization of the depolymerised samples.

Table 1 SI. Degree of deacetylation (DD) of low MW chitosan from various characterisation methods.

Method		%DD
Fluorescence	Fluorescamine assay[a]	6.0±0.4
UV-Vis	Ninhydrin test[a]	32.0±0.8
	Colloid titration[a]	91.2±0.5
^{1}H-NMR		91.1
IR (probe band/reference band)	1655/2876	87.5±1.0
	1560/2876	84.9±1.0
	1375/2876	75.1±1.0
	1310/2876	76.5±1.0
Elemental analysis	(C/N ratio)	84.1
Supplier data (Aldrich®)		75-85

[a] n = 3, expressed as mean ± SD

2. Degradation of chitosan

Table 2SI. Results of nitrous acid-induced depolymerisation of chitosan

MW of chitosan (kDa)	Sodium nitrite (mM)	Reaction time (h)	DD (%)		
			^{1}H-NMR	IR*	Colloid titration
478	0	0	91	85	90
234	2.5	0.25	92	88	88
97	2.5	1	91	89	88
51	2.5	3	92	87	86
36	2.5	12	91	89	84
23	5.0	12	92	88	82
13	7.5	12	90	83	75
9	10	12	82	83	72

* Calculated from the ratio of the absorbance at 1655cm^{-1} to that at 2876cm^{-1}

3. Cytotoxicity of chitosan samples

In this study, L929, a mouse connective tissue fibroblast cell line, was selected for *in vitro* cytotoxicity test, as recommended by USP 26.

The chitosan samples were prepared using equal volumes of 0.5% acetic acid chitosan solutions and double DMEM medium to produce solutions with chitosan concentration at 10 mg/mL, as described previously by Mao et al.[17]. The pH of the solution was adjusted to 6.5 with 1 N NaOH and the solution was further diluted when needed, using medium at pH = 6.5 as the solvent. L929 mouse connective tissue fibroblast cells were maintained in DMEM medium, supplemented with 10% foetal bovine serum (FBS), 2mM glutamine, 100unit/mL penicillin-streptomycin, at 37°C in a 5% CO2 humidified incubator.

MTT assay. Cells were obtained from exponentially growing cultures by trypsinization and were seeded at a density of 8,000cells/well into 96-well microtitre plates. Cells were incubated for 24h before the culture media were replaced with chitosan samples in a range of concentrations between 0.3 and 10 mg/mL. Following a 24 h exposure to chitosan, the tested solutions were removed by aspiration and the cells were washed twice with PBS. Then 100μl of 0.5 mg/mL MTT solution in culture media were added to each well. After the cells were incubated for 4h, the stain was removed by aspiration and 200μl of DMSO were added to each well to solubilise the formazan crystals. The plates were shaken for 5min before the absorbance at 550 nm was recorded, with a background correction at 690 nm. The cell viability was expressed as the ratio between the absorbance value of cells treated with chitosan samples and of control cells cultured in medium pH 6.5. The concentration at which 50% of cell growth is inhibited (IC_{50}) was obtained by interpolation from %cell viability and concentration plots (Figure 4 in the manuscript). Experiments were performed in six replicate wells for each chitosan sample and the cell viability was calculated and expressed as mean $\pm$ SD.

Live-Dead stain. Cells were seeded in 96-well microtitre plates, at a density of 4,000cells/well. Cells were incubated for 24h before the culture medium was replaced with 100μl of the chitosan solution at a concentration of 1mg/ml. After 24h exposure to chitosan, the tested solutions were removed by aspiration and the cells were washed three times with PBS. 100μl of the staining solution, from the LIVE/DEAD double staining kit, was added in each well and the cells were incubated at 37ºC for 15min before being observed under a fluorescence microscope (Leica DMI5000). The kit contained calcein-AM and propidium iodide (PI) solutions, which stain viable and dead cells, respectively. Both calcein and PI-DNA can be excited with 490 nm for simultaneous monitoring of viable and dead cells, while with 545 nm excitation, only dead cells can be observed.

Cell circularity (expressed as $4\pi(\frac{area}{perimeter^2})$, i.e. in a scale where 1 corresponds to perfect cricles) was measured through an ImageJ routine, using the fluorescence microscopy

pictures obtained for the live-dead stain test; the values (Figure 5 in the manuscript, top right) are averages calculated for at least 100 cells

4. Viscosity of chitosan solutions

Table 3SI. Viscosities of chitosan with different MW in varying concentrations and pH

$\overline{M_v}$ (kDa)	Concentration (wt. %)	pH	Dynamic Viscosity (mPa.s)	
			Mean*	SD
478	0.5	4	12.182	0.003
	1.0	4	41.716	0.147
	1.5	4	93.697	0.417
191	1.0	4	6.445	0.011
72	1.0	4	4.695	0.010
36	1.0	4	2.969	0.004
	1.0	5	2.608	0.002
	1.0	5.5	2.516	0.003
	1.0	6	2.457	0.005
	1.5	6	3.575	0.001
	1.75	6	4.124	0.001
	2.0	4	6.351	0.001
	5.0	4	36.61	0.314
23	1.0	4	1.735	0.004
	2.0	4	2.784	0.009
	5.0	4	8.244	0.005
9	1.0	4	1.067	0.006
	2.0	4	1.113	0.010
	5.0	4	1.423	0.003

* n =5

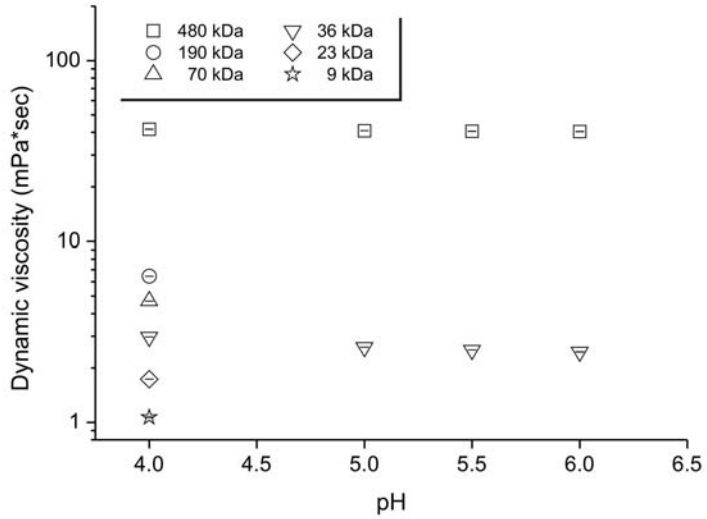

Figure 5SI. Dynamic viscosity of 1 % wt.f chitosan solutions as a function of chitosan molecular weight and of the pH of the 1 % wt. acetic acid solution (pH adjusted via addition of concentrated NaOH)

5. Preparation of chitosan/TPP spherical microparticles

Table 4SI. pH and concentrations used in the preparation of spherical microparticles.

Chitosan		TPP		Tween	
Conc. (% wt.)	pH	Conc. (% wt.)	pH	Conc. (wt %)	Average size (μm) ± SD
1	4	2.5	4	0	670 ± 170
1	4	2.5	4	1	640 ±150
1	5	2.5	5	0	660 ±150
1	5	2.5	5	1	590 ±120
1	5.5	2.5	5.5	0	570 ±140
1	5.5	2.5	5.5	1	690 ±110
1	6	2.5	6	0	620 ±110
1	6	2.5	6	1	640 ±130
1	5.5	2.5	7	1	660 ±140
0.5	4	1.25	7	0	Polydisperse aggregates
0.5	4	2.5	7	0	Polydisperse aggregates
0.5	4	5	7	0	Polydisperse aggregates
1	**4**	**0.8**	**7**	**0**	**420 ± 30**
1	4	1.25	7	0	410 ± 80
1	4	2.5	7	0	400 ± 60
1	4	5	7	0	400 ± 50
1.5	4	1.25	7	0	520 ± 130
1.5	4	2.5	7	0	550 ±160
1.5	4	5	7	0	500 ±90
1	5.5	2.5	7	1	660 ±140
1	4	2.5	8	0	480 ± 90
1	5.5	2.5	8	1	690 ±120

In this screening, the pH and concentration of the chitosan (acetic) solutions were varied in the range 0.5 – 1.5 wt. and 4 – 6, respectively, while those of TPP were varied in the range

0.5 – 5 % wt. and 4 – 8. In addition, Tween 20 was used in some experiments at a concentration of 1 % wt., to test whether a decrease in the surface tension of the TPP bath would have improved the chitosan droplet penetration in it, reducing the likelihood of coalescence of non perfectly gelled particles.

The optimized preparative conditions (in bold) were chosen on the basis of the minimization of the polydispersity of the microparticle dimensions, expressed above as the standard deviation of the microparticle diameter.

6. Characterization of chitosan/TPP microparticles

a. Calculation of composition on the basis of IR absorbance

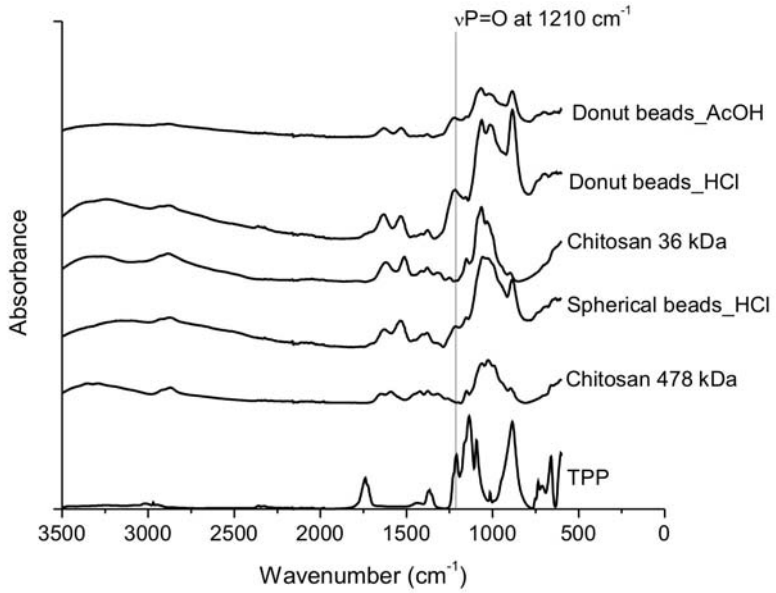

Figure 6SI. IR spectra for chitosan (two different molecular weights), TPP and several chitosan/TPP microparticles. The ratio of the intensity of the P=O stretching vibration, by the absorbance of the amide I vibration at 1655 cm^{-1} (chitosan) was used to assess the composition of microparticles. Also the ratio A(1210 cm^{-1})/A(2876 cm^{-1}) was used, resulting in markedly lower values (0.168, 0.341 and 0.342, respectively); this is an artefact, resulting from a much larger content of water (which artificially raise the absorbance at 2876 cm^{-1}) of the microparticles compared to the controls used for calibration.

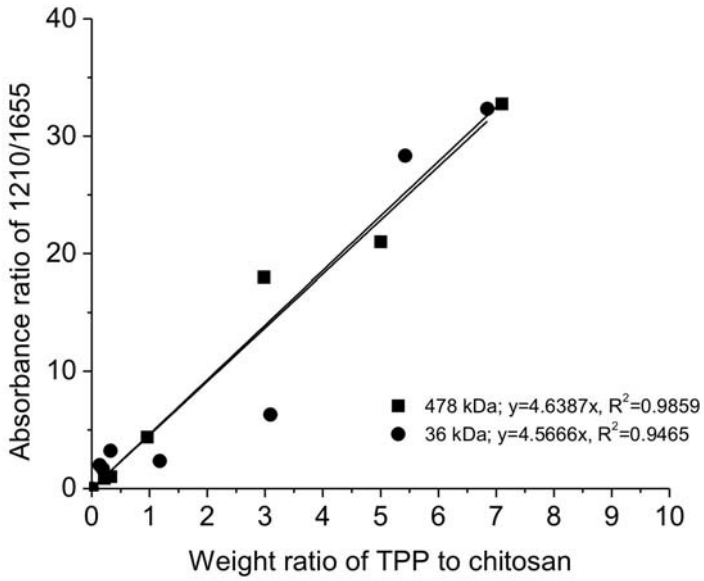

Figure 7SI. Absorbance ratio for the peaks at 1210cm^{-1} and at 1655cm^{-1} for mechanical mixtures of TPP and chitosan as a function of their weight ratio.

b. Sjze of "doughnut" microparticles

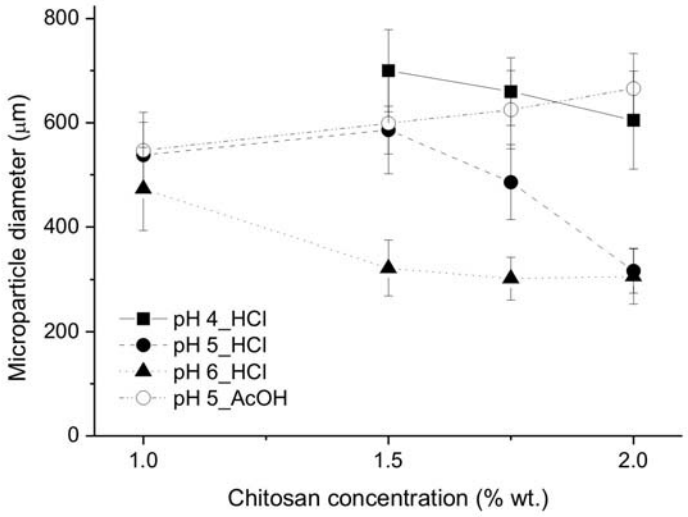

Figure 8 SI. Average size and standard deviation (samples of 80-100 microparticles) for toroidal microparticles produced from 1.5 % wt./pH = 6 36 kDa, chitosan and 0.8% wt./pH = 5 TPP

7. Effect of freeze drying on microparticle morphology

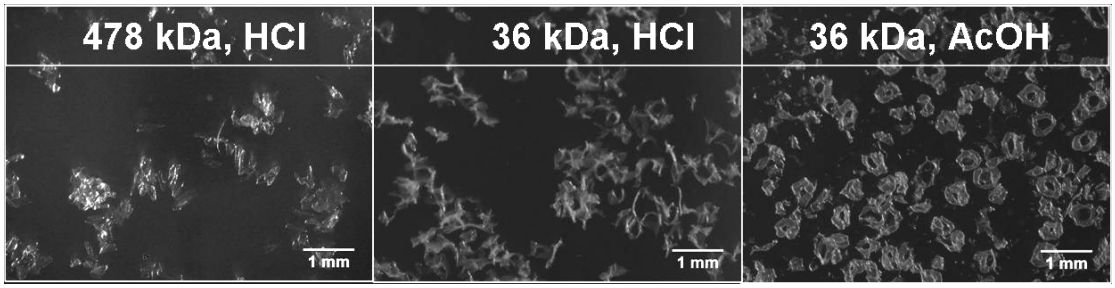

Figure 9 SI. Morphology of lyophylised microparticles after swelling in PBS for 24 hours. Only "doughnut" microparticles prepared from chitosan acetic solutions fully retained integrity and recovered a toroidal morphology.

8. References

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