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One-step sonochemical preparation of redox-responsive nanocapsules for glutathione mediated RNA release

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Abstract

Efficient RNA delivery to targeted cells requires the use of stable interactive carriers that provide RNA protection during the extracellular transit and trigger release once internalised. One strategy to avoid the premature extracellular RNA drain coupled to sufficient intracellular release is the use of stimuli-responsive delivery materials exploiting as a triggering mechanism the redox gradient between the extra- and intracellular compartments. This work describes a facile route for preparation of redox-active nanocarriers containing disulphides that combine RNA protection and delivery on demand based on intracellular glutathione (GSH) levels. A one-step sonochemical technology was employed to generate thiolated chitosan (TC) nanocapsules with diameter between 250 - 570 nm and simultaneously load them with RNA. Their size and physiological stability were directly proportional to the extent of disulphide cross-linking, which in turn could be ruled by adjusting the processing pH and degree of chitosan thiolation. The TC processing into nanocapsules showed to be advantageous in terms of RNA condensation and protection compared to the typically employed nanocomplexation. Fluorescence microscopy imaging revealed that: i) the nanocapsules enter the human fibroblasts and migrate to the perinuclear regions within 1 h, and ii) the cargo release may occur after the internalisation. These redox-responsive and biocompatible drug carriers demonstrated an effective (~60 %) and sustained (up to 72 h) RNA release at intracellular GSH concentrations (10 mM) *in vitro*, based on disulphide reduction and consequent capsules disassembly.

1. Introduction

Silencing of genes and non-functional proteins expression via delivery of small interfering RNA to mammalian cells is recognised as a versatile strategy to treat both inherited and acquired diseases.[1] Efficient RNA delivery requires the use of carriers to overcome many hurdles in order to release the intact cargo into target cells. A good carrier should provide: i) shielding from RNA degrading enzymes in body serum, ii) resistance to rapid RNA clearance from the blood stream, and iii) sufficient RNA internalisation to achieve the desired effect. Complexation of negatively charged RNA with highly cationic structures usually provides protection from degradation and rapid clearance, while promoting cellular uptake. Cationic carriers are selected among non-toxic, non-immunogenic and biodegradable polymers, e.g. biomacromolecules such as chitosan and its derivatives.[2] However, the high performance gene delivery is still compromised by a poor intracellular release from the cationic polymer/RNA complexes, leading to ultra-low transfection efficiencies.[3] Research efforts are currently focused on improvement of the transfection vectors based on their macromolecular structure that significantly affects their exploitation properties. For example, 3D molecule conformations such as cyclised knot polymer structure shows progress in gene delivery due to simplicity in synthesis, scalability, and high performance.[4] Other novel multifunctional gene delivery carriers are also under investigation, such as combination polycation-uncharged functional polymers that form unique ternary complexes with nucleic acids for more effective transfection.[5]

The use of stimuli responsive materials for triggered delivery inside cells without causing premature release is yet another attractive approach to overcome the low release. Temperature, pH, and redox gradient are examples of triggers inside the diseased cells that could be exploited with stimuli-responsive nanocarriers.[6-8] The redox-active tripeptide glutathione (GSH) is an endogenous reducing agent found in substantially

higher levels inside (1-11 mM)[9,10] than outside cells (0.002 mM in plasma).[11] Among other substrates, GSH acts as an electron donor to reduce disulphide bonds to free thiols.[12] The GSH-responsive intracellular cleavage based on -SH/-S-S- chemistry is thus exploitable to enhance the transfection capacity of disulphide-bearing vector systems.[8] In addition, conjugation of GSH itself with polymers brings about improvements in the binding to cell membranes and decondensed ability of nucleic acids from these systems in cytoplasm.[13]

Polymeric nano/micro-sized systems cross-linked with disulphide bridges can be assembled from a range of functional macromolecules to target triggered intracellular drug release.[14, 15] For example, particles of partially oxidised thiolated polymers are prepared using various techniques such as emulsification-solvent evaporation,[16] coacervation,[17] air jet milling,[18] and ionotropic gelation with Ca^{2+}.[19] However, most of these methods are based on a multi-step time-consuming processing that, in addition, offers little control over particle size and size distribution, thus rendering less predictable the interactions between the materials and targeted cells.

A facile one-step sonochemical route for generation of biopolymer-based nano/micro-capsules (NCs) has been proposed by Grinstaff and Suslick.[20, 21] By applying ultrasonic irradiation to biomacromolecular solutions supplied with air or at the interface of biphasic liquid mixtures, air-filled microbubbles or nonaqueous liquid-filled protein and polysaccharide NCs can be developed within minutes.[22, 23] Several disadvantages of this particle preparation technology were also identified, including higher variability in capsules size, their larger diameter (frequently in micrometer range) and encapsulation of extra phase, e.g. oil. For example, the method has been applied for the generation of a heterogeneous suspension comprising ~6 μM microbubbles and ~1 μM microcapsules prepared from thiolated macromolecules.[24] Although such large size distribution could

usually compromise the repeatability of the biological performance, these authors highlighted the sonochemically-induced intermolecular -S-S- cross-linking to improve the stability of the capsules in physiological fluids and envisaged the potential of the obtained systems in drug delivery applications. We have also recently employed the sonochemical technique for preparation of antibacterial thiolated chitosan (TC) nanocapsules that selectively kill *Escherichia coli* without imparting toxicity to human cells.[25]

The present work studies the feasibility of the sonochemical technology for generation of redox-responsive capsules comprising TC and their simultaneous loading with RNA. The process parameters were also optimised in order to achieve the human serum stable capsules with appropriate nanoscale size for fast cellular uptake. These biocompatible NCs should maintain their integrity and limit the RNA drain in oxidising environment, but release the cargo in the GSH rich *in vitro* medium.

2. Experimental section

2.1. Materials and reagents

Medical grade chitosan (Mw ca. 50 kDa, DDA 87 %) from Kitozyme (Belgium) was used for preparation of thiolated conjugates. Edible grade sunflower oil was used as organic phase for NCs preparation. Lysozyme from chicken egg white (111000 U/mg, 1 U corresponds to the amount of enzyme which decreases the absorbance at 450 nm by 0.001 per minute at pH 7.0 and 25 °C using *Micrococcus luteus* as substrate) was purchased from Sigma-Aldrich. For loading and release studies, the following RNA molecules were used: 1) total RNA from torula yeast (Sigma-Aldrich) as a model for small interfering (siRNA), and 2) Silencer® Cy™3 – siRNA (excitation max. = 547, emission max. = 563

nm), a 21-mer siRNA negative control labelled with Cy3 cyanine dye (siRNA-Cy3), especially designed for transfection studies (Ambion®, US). Thiolated chitosan labelled with fluorescein isothiocyanate (FITC, Sigma-Aldrich) together with siRNA-Cy3 was used for visualisation with fluorescent microscopy. Prior to thiolation, chitosan was labelled to obtained chitosan-FITC conjugate. Briefly, chitosan was dissolved in 1 % CH_3COOH to reach 2 mg/mL and pH was adjusted to 6 with 5 M NaOH. FITC (1 mg/mL) was dissolved in anhydrous DMSO. Then, to each 1 mL of chitosan solution 50 μL of FITC was slowly added and the reaction was incubated overnight at 4 °C in dark. The unbound FITC was separated from the chitosan-FITC conjugate using PD-10 Desalting Columns (GE healthcare). Unless specified otherwise, all other reagents and buffers were of analytical grade, purchased from Sigma-Aldrich, and used as received.

Synthesis of thiolated chitosan

TC synthesis was carried out in a one-step coupling reaction between 2-iminothiolane HCl and primary amino groups of chitosan and chitosan-FITC, according to.[26] Batches differing in the thiolation degree were prepared altering the amount of the coupling reagent in the reaction mixture. The thiolated conjugates (TC and TC-FITC) were named according to the chitosan:2-iminothiolane HCl weight ratio: $TC_{10\text{-}1}$ for 10:1 ratio, $TC_{5\text{-}1}$ for 5:1 ratio and $TC_{5\text{-}2}$ for 5:2 ratio, as previously reported.[27]

2.2. Preparation and characterisation of nanocapsules

Generation of TC nanocapsules

TC NCs were prepared in a one-step sonochemical process from a two-phase mixture containing 30 mL of TC aqueous solution (1 mg/mL) and 12 mL of sunflower oil. The pH of the aqueous phase was adjusted to 3, 4.5 or 6. The ultrasonic horn was positioned

at the aqueous-organic interface and the mixture was sonicated at 17 W for 3 min with the amplitude of 30 %. During the sonication the temperature was maintained 4.0±0.5 ºC using ice bath. The resulted emulsion was centrifuged at 2500 rpm for 15 min in order to complete the phase separation and the non-encapsulated oil was removed from the formulation. Together with the oil phase, larger NCs (above 1 μm) obtained during sonication were also removed by centrifugation (larger NCs filled with oil are found in the upper part of the suspension, close to the water/oil interphase). To prepare RNA loaded NCs, TC were sonicated in presence of RNA (100 μg/mL) and TC-FITC with siRNA-Cy3.

Characterisation of TC nanocapsules

The formation efficiency of the capsules was measured after filtering the obtained suspensions through Millex® syringe driven filter units with pore diameter 0.22 μm (Merck, Spain) in order to separate the capsules from the aqueous solution that contained non-encapsulated conjugates. The solutions were thereafter freeze-dried and the recovered conjugates were weighed (Wa) and compared to the starting weight (Wi=30 mg) prior to sonication. The percentage of the formation efficiency was defined as the weight ratio between the encapsulated conjugate (Wi-Wa) and the starting weight (Wi). All the measurements were carried out in triplicate and the results expressed as mean values.

The amount of reduced thiols in the NCs suspensions and the starting conjugates were determined spectrophotometrically at 450 nm using Ellman's reagent.[28] Prior to the absorbance measurements the suspensions were freeze-dried, re-dissolved in 0.5 M phosphate buffer (PB), pH 8, containing Ellman's reagent, and filtered to obtain clear solution for the measurement.

The NCs obtained from TC-FITC and siRNA-Cy3 were visualised using fluorescence microscopy. Appropriate filter sets were employed to record the images with a Nikon Eclipse Ti microscope, using a 100x oil-immersion objective. Visualisation was also conducted using scanning electron microscope (JSM-840, JEOL).

The NCs size was determined by dynamic light scattering (DLS) using DL135 Particle Size Analyzer (Cordouan Technologies, France). Three samples of each TC NCs suspension were processed acquiring 5 measurement cycles with 1 % signal-to-noise ratio. The data were analysed using NanoQ 1.2.1.1 software.

The determination of ζ-potential was carried out using a Zetasizer Nano Series (Malvern Instruments Inc., Worcester, UK) after appropriate dilution of suspensions using ultra-pure water obtained with a Milli-Q plus 185 from Millipore Ibérica S.A. (Spain).

The stability of TC NCs to enzymatic degradation was studied in a biodegradation test using lysosyme. NCs suspensions (1 mL) were incubated with 1 mL of 50 mM PB, pH 6.6, in the presence of 10 μg/L lysozyme at 37 °C for 24 h. Aliquots of 250 μL were thereafter withdrawn and filtered in order to remove the intact capsules from the filtrate. The amount of the degraded conjugate in the filtrate was estimated based on amine group determination using ninhydrin test[29] and expressed in mM of glycine equivalents, used as a standard to build the calibration curve.

2.3. RNA loading into TC nanocapsules

RNA loading capacity

The ability of the sonochemically processed TC NCs for RNA loading was assessed in terms of their condensation capacity (using a gel retardation assay) and loading efficiency (using a fluorimetric method). For gel electrophoresis, the samples were loaded onto 2 % (w/v) agarose gel and the analysis was carried out at 80 V for 60 min, running with Tris-

acetate-EDTA buffer (TAE). Ethidium bromide was used to visualise RNA bands using a UV transilluminator imager (Chemi Doc™ MP, Bio-Rad, USA). On the other hand, the RNA loading efficiency (%) was determined after the suspension filtration and 1:100 dilution in Tris-EDTA buffer (TE) using the fluorimetric Quant-iT™ RiboGreen® RNA Assay Kit (Life Technologies, Spain).

RNA stability within TC nanocapsules

RNA containing TC NCs were incubated at 37 °C with equal volume of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20 % of fetal bovine serum (FBS). At each predetermined time point (0, 1, 6 and 24 h) 200 μL of the suspension was collected and the serum activity was terminated by raising the temperature to 80 °C for 5 min. Thereafter, the RNA was recovered by phenol/chloroform extraction method[30] and its integrity was analysed by gel electrophoresis.

2.4. Cytotoxicity assay and cellular uptake of nanocapsules

Cell culturing

For cell culturing human foreskin fibroblasts cell line BJ-5ta (ATCC-CRL-4001, LGC Standards S.L.U., Spain) at passage 7 were used. The cells were maintained in 4 parts Dulbecco's Modified Eagle's Medium (DMEM, LGC Standards S.L.U) containing 4 mM L-glutamine, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, 1 mM sodium pyruvate and 1 part of Medium 199, supplemented with 10 % (v/v) of fetal bovine serum (FBS), and 10 g/mL Hygromycin B at 37 ºC, in a humidified atmosphere with 5 % CO_2. The culture medium was replaced every 2 days. At pre-confluence, cells were harvested using trypsin-EDTA (0.25 % (w/v) trypsin/0.53 mM EDTA solution in Hank's BSS without calcium or magnesium, LGC Standards S.L.U.).

Biocompatibility studies

Cells were seeded at a density of 4.5 x 10^4 cells/well on a 96-well tissue culture-treated polystyrene plate (Nunc, Spain) the day before experiments and then exposed to 150 µL NCs suspension diluted in DMEM (1:10 v/v). Cells and NCs were incubated at 37 ºC in a humidified atmosphere with 5 % CO_2 for 24 h. Thereafter, the incubated cells were examined after 72 h for signs of toxicity using Alamar Blue assay. Resazurin, the active ingredient of AlamarBlue® reagent (Life Technologies Corporation, Spain), is a non-toxic, cell-permeable compound that is blue in colour and reduced to resorufin by viable cells, developing a red colour compound. After contact with cells, the medium with NCs was removed and the cells were washed 2 times with PBS. AlamarBlue® (100 µL of 10 % (v/v) in DMEM) was added and after 4 h of incubation at 37º C, the absorbance at 570 nm was measured, using 600 nm as a reference wavelength, in a microplate reader Infinite M200 (Tecan, Austria). The quantity of resorufin formed is directly proportional to the number of viable cells. BJ5ta cells relative viability (%) was determined and compared to that of cells incubated only with cell culture medium. As a positive control of cell death, 500 µM H_2O_2 was used. All tests were performed in triplicate.

TC NCs internalisation and intracellular distribution

The cellular uptake of the sonochemically produced NCs and their intracellular distribution was evaluated using fluorescent microscopy. To allow the NCs visualisation, a lipophilic dye Nile red was solubilised in oil (0.01 mg/mL) and encapsulated together with the oil phase during the sonochemical processing. Cells were seeded at a density of 5.6 x 10^4 cells/well on a 12-well tissue culture-treated polystyrene plate (Nunc) the day before experiments and then exposed to 1 mL NCs suspension diluted in DMEM (1:1 v/v) at 37 °C for 24 h. Thereafter, the medium with NCs was removed and the cells were

washed 2 times with PBS. The cell nuclei were finally stained with Hoechst 33258. The collected fluorescence was passed through three filters: DIC, DAPI and TRITC. Appropriate filter sets were employed to record the images with a Nikon Eclipse Ti microscope, using a 40x objective.

2.5. GSH-mediated RNA release from TC nanocapsules

TC NCs loaded with RNA were incubated in PBS supplemented with 2 μM and 10 mM GSH at 37 ºC at a 1:9 ratio (v/v). At different time intervals the mixtures were filtered and the filtrate solutions were quantified for RNA content after appropriate dilutions in TE buffer using Quant-iT™ RiboGreen® RNA Assay Kit.

Reduced thiols after the NCs incubation with GSH were determined using Ellman's reagent. Since the GSH thiol would also reduce -S-S- from the reagent to develop colour, prior to its addition the suspensions were precipitated in ethanol:water mixtures (ratios 9:1 and 1:1) to remove non-reacted GSH from the chitosan conjugate. The samples were freeze-dried, re-dissolved in PB (pH 8) containing Ellman's reagent and filtered, before the absorbance was measured at 450 nm.

3. Results and discussion

3.1. Synthesis and characterisation of TC nanocapsules

Ultrasonic irradiation of two-phase liquid mixtures produces both emulsification and acoustic cavitation – the formation, growth and implosive collapse of micron-sized bubbles.[31] Creating the possibility of chemical reactions around the formed bubbles, this technique is successfully employed for generation of NCs from proteins and polysaccharides. Sonication at the interface of a biopolymer aqueous solution and organic

solvent results in formation of core-shell biopolymer capsules filled with nonaqueous liquids. In this research, a 3 min sonochemical process at pH ranging from 3 to 6 was used to formulate oil-filled TC NCs. After centrifugation and removal of larger particles, SEM imaging revealed generation of spherical sub-micron capsules for all experimental groups (Fig. 1). The images also suggested higher density of the capsules and a general pattern of smaller structures in the suspensions obtained at higher pH.

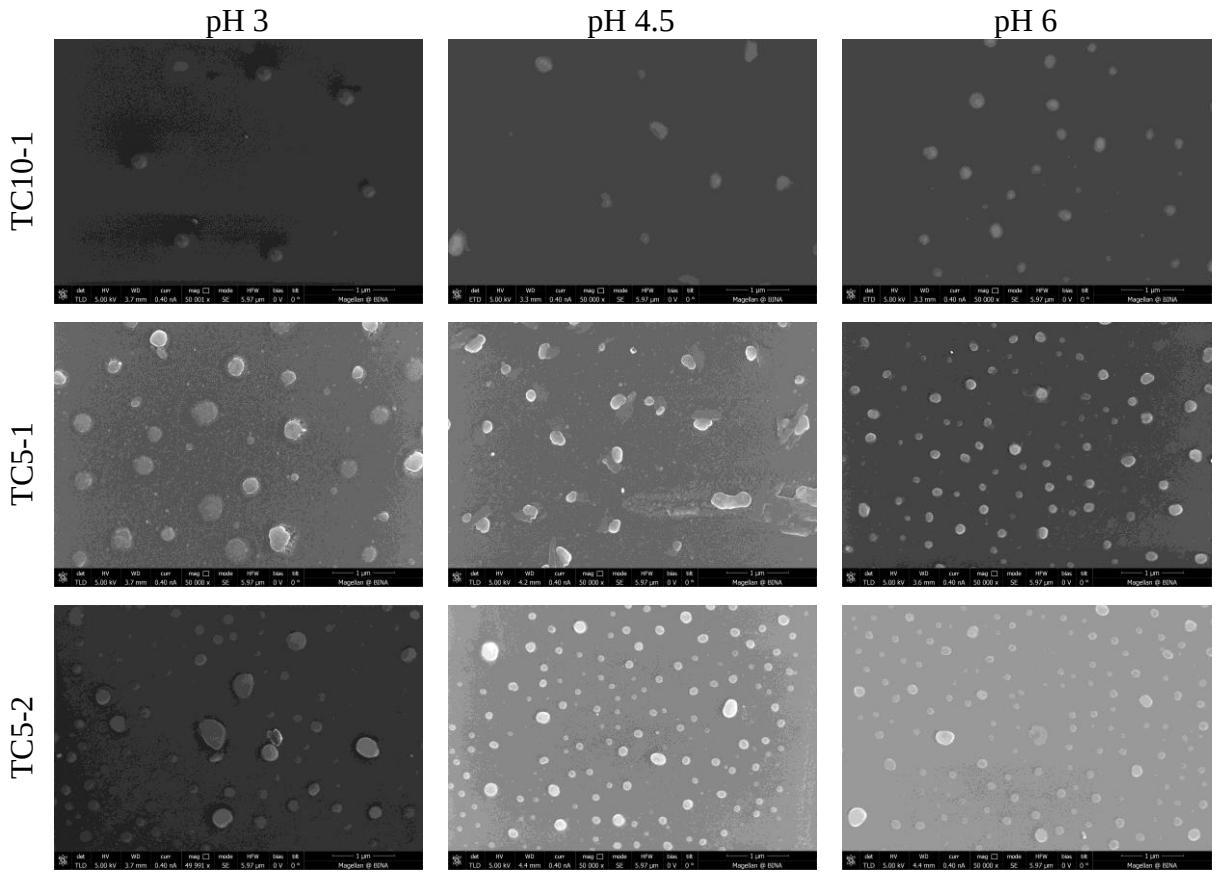

Figure 1. SEM images of sonochemically generated TC NCs (MAGX50K)

The formation efficiency of the capsules indeed increased with the processing pH and the conjugate thiolation degree (Table 1). On the other hand, the DLS characterisation also corroborated the SEM findings, where the mean capsule diameter could be tuned by adjusting the above two parameters (Table 1). The range of the capsules size was from

570 nm for TC_{10-1} processed at lower pH values down to 253 nm for TC_{5-2} obtained at pH 6. It is worth mentioning that the mean NCs diameter and the visual appearance of the suspensions did not change even after three months storage, revealing remarkable physical stability of these systems. A reason for such stability may be the high cationic character of the capsules, evaluated by ζ-potential measurements (Table 1). Moreover, the size distribution of the capsules was narrower if the suspensions were processed at higher pH (Fig. 2). This is especially visible in the TC_{10-1} NCs sample processed at various pH values.

Table 1. Characterisation of TC NCs

NCs suspension	Formation efficiency (%)			Mean NCs size (nm)			ζ-potential (mV)
	pH 3	pH 4.5	pH 6	pH 3	pH 4.5	pH 6	pH 4.5
TC_{10-1}	64±10	63±12	74±6	570±13	570±16	357±21	+27.9±1.2
TC_{5-1}	72±7	82±7	89±7	502±19	399±34	262±22	+28.9±1.7
TC_{5-2}	76±9	93±10	92±9	506±36	265±09	253±20	+30.3±2.1

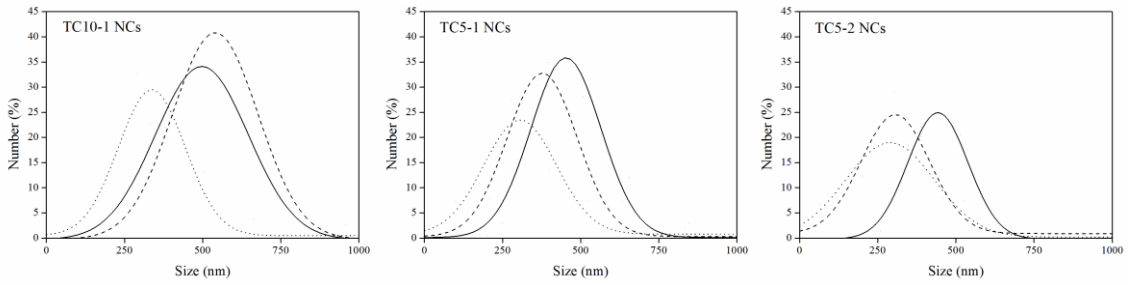

Figure 2. Size distribution of TC NCs measured by DLS. The capsules are processed at pH 3 (full line), pH 4.5 (dashed line) and pH 6 (dot line).

Since the mean size of polymer particles usually decreases after chemical cross-linking, our data indicate the -SH oxidation to disulphides and consequent capsules cross-linking under sonication.[32] To determine the extent of -SH/-S-S- conversion, the free thiol content in the capsules is compared to their respective controls, i.e. thiolated conjugates before sonication. All suspensions displayed less reduced thiols per gram of formulation regardless of the processing pH (Fig. 3A). In addition, the extent of oxidation was pH dependent: whereas all formulations sonicated at pH 6 contained negligible amounts of reduced thiols, only between 53 - 72 % were oxidised at pH 3, where the extent depended on the -SH amount in the starting conjugate.

Indeed, during the ultrasonic irradiation reduced thiols are typically oxidised to disulphides by the superoxide radical,[21] which ultimately leads to microencapsulation.[24] The -SH/-S-S- redox transition is further promoted at higher pH and considerably faster in thiol-rich systems,[33] which herein findings confirm. Although recent studies revealed that -S-S- cross-linking is not critical for the capsules formation,[34] these bonds undoubtedly play an important role in the particles stabilisation.[16] Our TC NCs with different extent of cross-linking were therefore tested for stability against lysozyme degradation, a common enzyme in human body fluids able to hydrolyse chitosan-based materials. The NCs processed at pH 3 and pH 4.5 showed higher degradation yields than those processed at pH 6 (Fig. 3B). The capsules stability was also influenced by the thiolation degree of the starting conjugate, where $TC_{5\text{-}2}$ NCs suffered the lowest degradation rates regardless of the processing pH.

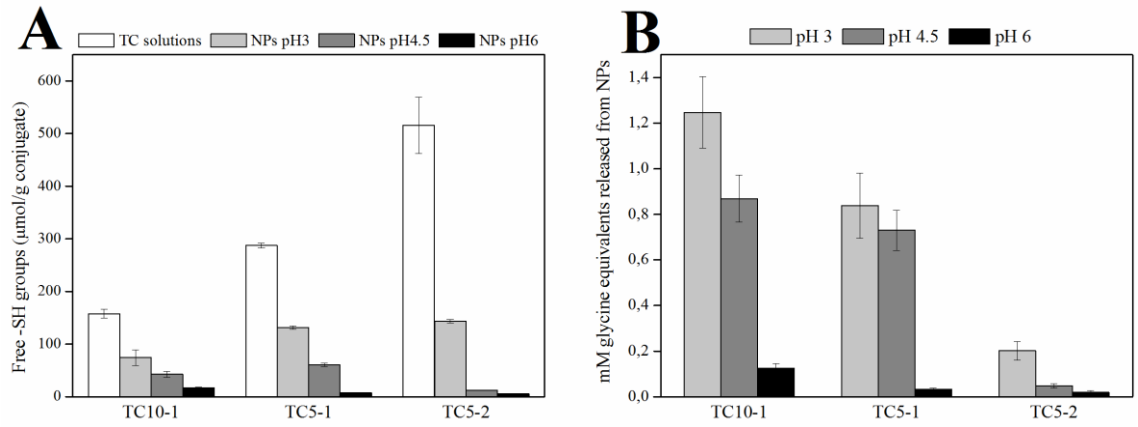

Figure 3. Correlation of the: A) -SH content and B) resistance to lysozyme degradation of the TC NCs.

Cross-linking of any biopolymer-based carrier, e.g. through -S-S- formation, targets improvement of its physiological stability, protection of unstable active compounds and their sustained release.[35] On the other hand, the presence of reduced thiols amplifies the functional potential of a biomaterial, including improved mucoadhesion for prolonged pharmacological effects, permeation enhancement, or inhibition of targeted enzymes.[36] Optimal thiol/disulphide ratio in biocarriers is therefore driven by the specific application and the -SH oxidation has to be controlled during the preparation protocol. Our study demonstrated that the -SH/-S-S- ratio in the sonochemically generated NCs is adjustable by controlling the processing pH and thiolation degree of the starting conjugate, which allows easy tuning of their functional properties. In order to avoid a large number of experimental groups, all further studies were conducted only with $TC_{5\text{-}1}$ NCs formulation containing RNA processed at pH 4.5 (in further text referred as TC/RNA NCs).

3.2. RNA loading into TC NCs and its stability in serum

RNA loading into TC NCs was first evaluated using fluorescence microscopy. For the purpose of better imaging, the sonochemically obtained TC/RNA NCs suspension was

not centrifuged and the NCs larger than 1 μm are herein also visualised. These analyses confirmed the SEM findings of spherical in structure capsules (Fig. 4). The FITC green fluorescence signal was located only in the outer part of the capsules (Fig. 4B), indicating that the NCs shell consists of the TC component. In contrast, the siRNA-Cy3 red signal was mainly located in the capsules core (Fig. 4C and 4D), revealing the RNA encapsulation within the TC capsules. Thus, the large part of the RNA molecules are distributed in the capsules core, with very few found in the shell, which is in a good agreement with the previous similar works.[37] These images confirm that a 3 min sonochemical process was sufficient to formulate thiolated chitosan capsules and simultaneously encapsulate RNA. Importantly, the RNA encapsulation did not lead to a significant size alteration, as the loaded NCs displayed the mean size of 407±54 nm compared to 399±34 nm of those without RNA (DLS measurements were carried out after centrifugation).

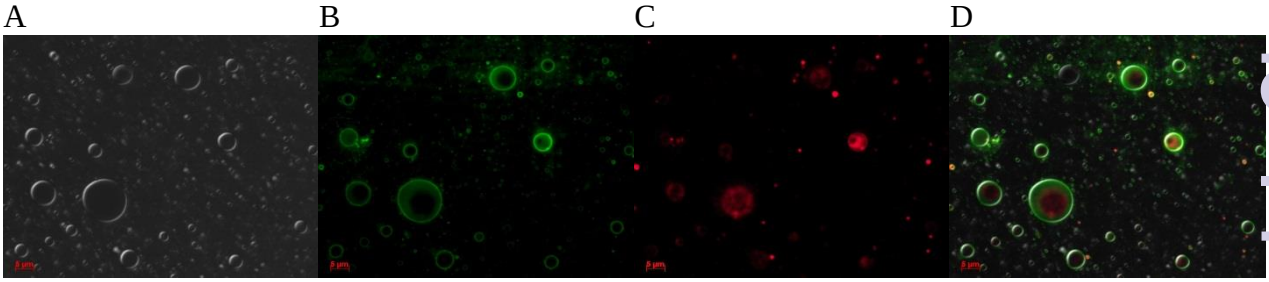

Figure 4. Fluorescence microscope images of sonochemically generated TC/RNA NCs: A) differential interference contrast image, (B) green fluorescence mode (λexc= 489 nm), (C) red fluorescence mode (λexc= 543 nm), (D) merge mode.

The ability of TC NCs for RNA loading was also evaluated by an agarose gel retardation assay. For comparison, naked RNA, TC/RNA nanocomplexes and pure RNA NCs were run in parallel. TC/RNA nanocomplexes were processed at pH 6 by vortexing the mixture containing the same starting concentrations of TC and RNA,[38] whereas RNA NCs were

processed sonochemically using the previously described method.[39] Whereas the RNA mobility in TC/RNA nanocomplexes was substantially retarded, but still detectable, the movement of TC/RNA NCs was entirely retarded (Fig. 5A, agarose gel). The disappearance of the band in the latter indicates the complete loading of RNA. Such observation was confirmed by the fluorimetric measurements for loading quantification, where negligible amounts of RNA were detected in the filtrate of the TC/RNA NCs suspension (Fig. 5A, bottom graph). On the other hand, up to 79 % of RNA was loaded into TC/RNA nanocomplexes. Thus, the sonochemical method for preparation of NCs resulted in more efficient RNA condensation compared to the conventional complexation approach. No differences in the nucleic acid mobility for the naked RNA and RNA NCs were observed (Fig. 5A, agarose gel). This could be expected since the sonochemical spherisation does not lead to degradation of RNA,[39] and RNA NCs are biologically active and equally recognised by RNA kinases as the intact RNA. Importantly, the differences in the migration of the naked RNA and RNA NCs on one side, and TC/RNs NCs on another, together with the absence of the RNA band in the latter, implies that no RNA nanocapsules were presented in the TC/RNA NCs suspension, confirming the findings of fluorescence microscopy. In other words, the shell of TC/RNA NCs is entirely built of thiolated chitosan that encapsulate the intact RNA molecules. Such structure represents the most promising way to increase the serum stability of nucleic acids without compromising their efficiency.

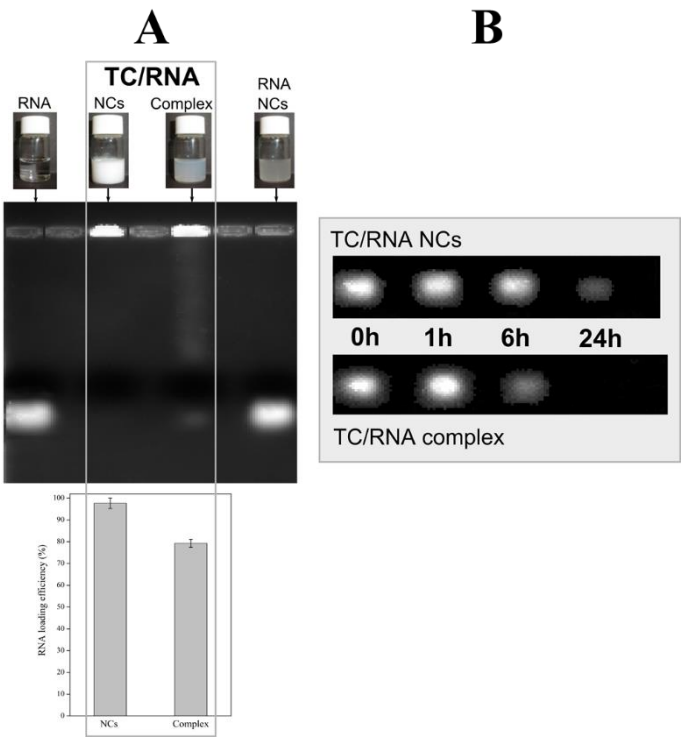

Figure 5. RNA loading onto TC nanocapsules and stability in serum: A) agarose gel electrophoresis (retardation assay) and fluorimetric assay to determine RNA condensation and loading efficiency by TC NCs, and B) protection of RNA in TC/RNA NCs and TC/RNA nanocomplexes from serum nucleases (20 % FBS).

The RNA stability was further tested against serum nucleases degradation. A rapid degradation of naked RNA (within 1 h) and pure RNA NCs (after 1 h) was observed (results not shown). By contrast, the degradation from the TC/RNA nanocomplexes was slower and occurred only after 6 h (Figure 5B). These findings were in a fair agreement with the RNA degradation from similar nanocomplexes.[40] Nevertheless, higher RNA loading into TC/RNA NCs compared to the nanocomplex was also translated into improved stability to nuclease degradation, as the RNA from NCs was not fully degraded even after 24 h of incubation. Since no RNA capsules were presented in the TC/RNA

NCs system it could be concluded that such stability was entirely due to the formation of TC nanocapsules.

3.3. Biocompatibility and cellular uptake

Prior to the internalisation studies, the effect of TC/RNA NCs on human fibroblasts viability was evaluated using AlamarBlue® assay composed of a resazurin dye that is reduced by cellular reductases to a pink-coloured chromophore – a direct measure of cell viability.[41] The TC/RNA NCs had negligible cytotoxic effect, with cells showing 96.1 ± 1.5 % viability after 72 h contact, relative to the control without the NCs. Fluorescence microscopy images further showed that the Nile red core-labelled NCs were readily up-taken by the fibroblasts (Fig. 6). The capsules efficiently penetrated the membrane and entered the cells within 1 h, which confirmed the general compliance that the uptake of structures with less than 500 nm mean size is more facile than that of micro-sized materials.[42] Nevertheless, nanoscale size and spherical shape alone are not sufficient to favour the efficient cellular uptake of these NCs. High positive surface charge (Table 1), i.e. the cationic character of these NCs should be considered the main driving force for effective interaction with the negatively charged cell membrane and facile internalisation.[43] It is well established that NCs efficiently internalise by pinocytosis involving four different pinocytic mechanisms or their combinations.[44] Clathrin-mediated pathway seems to be the preferred way for internalisation of cationic systems and manufacturing of nanoparticle carriers is advancing in that direction.[45, 46] Such investigation, however, goes beyond the scope of this manuscript.

The microscopy images also revealed a wide intracellular fluorescence distribution, which may suggest the release of Nile red from the internalised capsules. Release of cargos has been previously related with reducing disulphide linkages in the polymeric

vehicles.[15] According to this hypothesis, sonochemically generated TC NCs appear as promising carriers for non-invasive intracellular drug administration. Further staining of the DNA from the fibroblasts nuclei with Hoechst 33258 facilitated the intracellular tracking of the internalised capsules. Overlapped phase contrast/fluorescence images revealed that the capsules/dye were dispersed throughout the cytoplasm, migrating to the perinuclear region of the cells within 1 h. Their concentration around the nucleus, rather than near the cellular membrane, indicates the rapid trafficking of the capsules through the cytoplasm.[47] Expectedly, there was no colocalisation of the capsules in the nucleus, as the transport through the nuclear pore complexes occurs for particles with size up to 39 nm.[48]

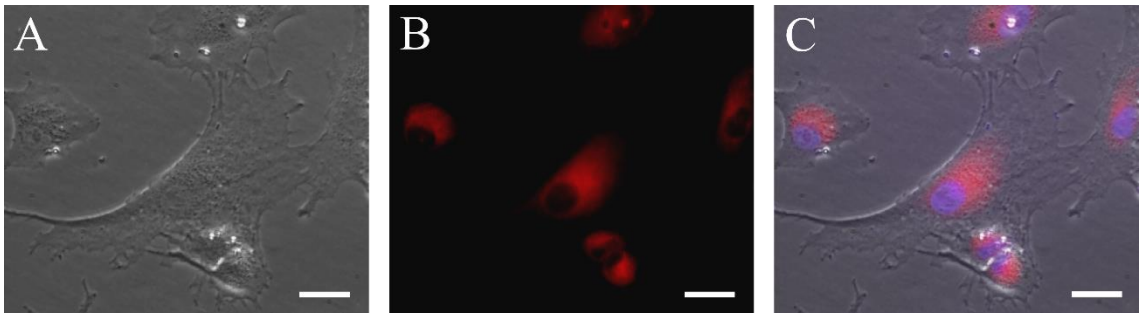

Figure 6. Interaction of TC/RNA NCs with BJ-5ta fibroblasts: microscopy images showing the intracellular distribution of the capsules: A) phase contrast image of cells, B) fluorescent microscopy image of the capsules containing Nile red inside cells, and C) the overlay image with nuclei stained with Hoechst 33258. Scale bars: 25 μm.

Disulphide linkages have been associated with efficient nucleic acid delivery in almost all vector systems tested so far.[49] Due to their enhanced interaction with cellular membranes, oxidised thiols induce improved internalisation in various cell types. In addition, these are able to provide enhanced intracellular release of nucleic acids based on high GSH levels.[50-52] However, emerging evidences also demonstrate that not only -S-S- but also -SH containing structures could effectively cross-react with cell-surface and

enhance the cellular uptake of a drug.[53] During such internalisation pathway cell-surface thiols are responsible for the partial entrapping of the -SH/-S-S- systems in the membrane[54] and further drug uptake via an extracellular mechanism, instead of widely accepted GSH-mediated cargo release. With the increasing number of conflicting reports, an effective intracellular delivery of many active agents most probably combines both extracellular and intracellular pathways.

3.4. TC NCs exhibit reduction triggered RNA release

Redox gradient between the extra- and intracellular compartments, due to the difference in GSH levels, is the biological rationale for synthesis of redox sensitive, disulphide-based carriers for intracellular drug delivery.[8] In order to determine the RNA release patterns from sonochemically prepared TC/RNA NCs *in vitro*, the formulation was incubated with PBS containing extracellular (2 μM) and intracellular GSH (10 mM) levels . Our results showed that RNA was progressively released from the NCs, where the released amount strongly depended on the GSH concentration (Fig. 7A). Slow release pattern was detected in the suspension with 2 μM GSH, reaching 19 % of the total RNA loaded, whereas the release was markedly improved in the presence of intracellular GSH levels (62 %). In the latter case the RNA was released over a period of 72 h, which means both enhanced and sustained RNA delivery. It is logical to assume that the high GSH concentration used in this study induces effective reduction of disulphides in TC/RNA NCs[55] and, consequently, improved RNA release. To support this assumption, the -SH content in the mixtures was determined after incubation with 2 μM and 10 mM GSH. In addition, photographs of the mixtures were also taken at each evaluation point for visual examination of the macroscopic changes in the suspensions. Since the -S-S- bonding was the primary mechanism of the capsules formation and stabilisation, it is expected that

upon their cleavage the particles will re-solubilise. Indeed, almost clear solution was obtained after 72 and 96 h incubation with 10 mM GSH, whereas no visual changes were detected in the suspension incubated with 2 μM GSH (Fig. 7B). Accordingly, after 72 h incubation in GSH rich medium the amount of reduced thiols in the mixture increased by 203 μmol g^{-1}, compared to only 33 μmol g^{-1} in presence of low GSH levels (Fig. 7C). Thus, the reduction of disulphides to destabilise TC/RNA NCs and to consequently induce RNA release were GSH dose-depended at the concentrations found in extra- and intracellular compartments.

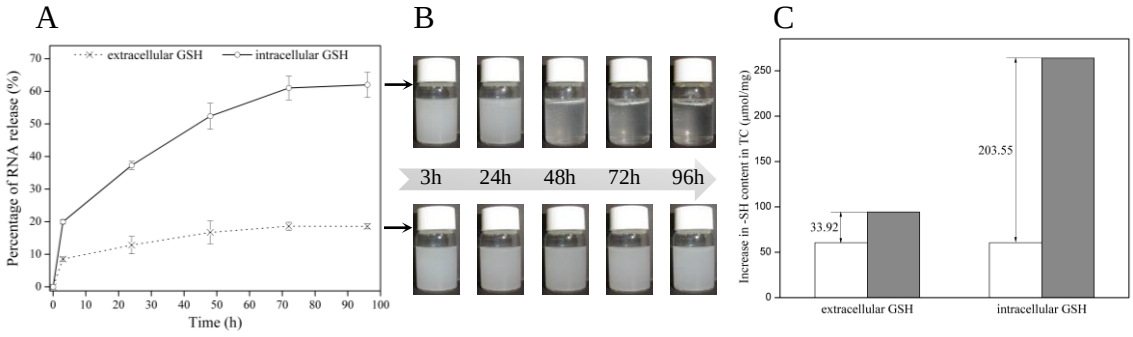

Figure 7. A) Release of RNA from TC/RNA NCs in presence of 2 μM and 10 mM GSH; B) photographs of TC/RNA NCs degradation by 2 μM and 10 mM GSH; C) increase in reduced thiol content in the mixtures treated with 2 μM and 10 mM GSH (white bars – initial -SH content; grey bars – content of -SH after incubation with GSH).

The herein obtained periods for RNA release are rather long compared to the reported up to 24 h release *in vitro* for redox sensitive systems based on complexing of -SH and -S-S- modified chitosans with nucleic acids.[38,55,56] Our results are in agreement with sustained release patterns from sonochemically generated biopolymer capsules.[57] Overall, the rationale for ultrasound-assisted preparation of -S-S- cross-linked NCs for effective

entrapping of nucleic acids is also justified with a sustained cargo release and potentially prolonged beneficiary effect compared to self-assembled nanocomplexes (the duration effect will be evaluated in future studies involving *in vitro* and *in vivo* transfection experiments).

4. Conclusions

In this research the feasibility of a one-step sonochemical technology to generate redox-responsive nanocapsules and simultaneously load them with RNA was assessed. A facile and reproducible sonication process resulted in formation of disulphide containing RNA carriers starting from thiolated chitosan, which otherwise could be achieved after performing time and chemical consuming cross-linking operations. The short sonication time (3 min), sufficient to obtain physiologically stable nanoscale capsules without any stabilisers, renders this technology attractive for pharmaceutical processing. The study also achieved to optimise the process variables highlighting the importance of the processing pH and chitosan thiolation degree on the extent of disulphide cross-linking and nanocapsules stability. These in turn exhibited remarkable RNA loading capacity, long-term RNA protection from serum nuclease degradation, biocompatibility, and fast cellular uptake. Disulphide cleavage and consequent capsules disassembly at the intracellular GSH levels was a trigger for enhanced, but prolonged (up to 3 days), release of RNA.

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