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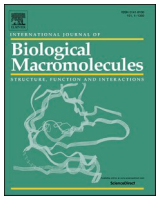

Nano spray-dried particles of in-situ crosslinked alginate and their toxicological characterisation

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ABSTRACT

The feasibility and technical capacity for producing crosslinked sub-micron gels with a nano spray-dryer were studied with variable pH systems incorporating alginate, pectin, and pullulan. The obtained powders were characterized for their morphology, particle size distribution, and their toxicological safety profile using genotoxicity and cytotoxicity assays. Additionally, quercetin was added to the encapsulation system to study the potential of the system to encapsulate this material. The produced powders exhibited morphologies and particle size distributions characteristic for nano spray-drying. The addition of pullulan and pectin to the feed solutions resulted in a particle size increase, with crosslinked alginate particles having a mean value of 1.43 μm, while particles with added pectin and pullulan had a mean particle size of 1.70 and 1.75 μm, respectively. The inclusion of quercetin proved to be problematic with this encapsulation system. Extremely high degradation rates and extremely low encapsulation efficiencies were observed due to the alkaline pH (~10) of the system that is needed to keep the feed dispersion in a liquid state and prevent premature crosslinking of the alginate. Although pectin and pullulan provided some protection for quercetin in the alkaline dispersion, the absolute quercetin content in the final product remained very low, with a maximum achieved encapsulation efficiency of 2.06 %. The safety profile of most produced powders was favourable, as they did not exhibit any significant cytotoxic and genotoxic activity in the HepG2 cell line, except in the case of Alginate/Pullulan which showed a 43 % decrease in cell viability at 500 μg/mL. Samples where quercetin was added did not show any increased toxicological effect.

1. Introduction

The production of sub-micron particles on an appreciable scale is a challenge, especially in the field of dry powders with a spherical morphology. Production methods for defined nanoparticles typically rely on nano-precipitation [1] or templated self-assembly techniques [2], whereas submicron spray drying has become available relatively recently. In the last decade, progress has been made in nano-spray drying. Spray-dried particles on the sub-micron scale have become available due to the introduction of the piezoelectric spraying mesh system, i.e. a sonicating nozzle for droplet size decrease, as well as an electrostatic filtering unit at the outlet that enables harvesting of the particles that avoid separation by inertia. The technology to produce these sub-micron particles with a spray dryer comes with certain issues, namely the properties of the powders themselves and their handling. Sub-micron particles or particles of a few microns in size tend to penetrate certain mucosal tissues rather effectively [3,4]. As such they present a potentially useful pathway for the delivery of bioactive compounds into the body, especially in contact application or through mucous tissues, such as by inhalation of such powders. On the other hand, such particles may present a hazard to those handling them, as they do not sediment well, and they tend to linger in the air. As such they can be inadvertently inhaled, potentially causing harm [5]. Sub-micron particles contained within a dispersion are safely suspended within a dispersion medium, but can still present a risk in contact. The health effects of small persistent particles is typically dependent on their size, morphology, surface chemistry, and exposure route [5,6]. Nanoparticles for example have a larger surface area per mass than microparticles, thus

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leaving a larger surface area for potential interactions between the nanoparticle and cellular components [7]. There is no general rule on distinguishing a morphology that is potentially more hazardous as compared to others, and the methodology of this evaluation is not standarised. Therefore, individual considerations have to be made with regard to specific materials. Natural polysaccharides are frequently used for nanoparticle fabrication due to their biocompatibility, biodegradability, and functional versatility. For example, (nano)cellulose, is a material that is harmless on a macro scale, yet nanosized particles have been shown to cause certain, negative, immunological responses in cell models [8]. Even more so, we can observe the influence of morphological differences between two distinct cellulose nanomaterials, i.e. cellulose nanocrystals and nanofibrils, where one causes an inflammatory response and the other has genotoxic properties, respectively [8]. Yet the results of such tests are also highly dependent on the source of the material, the production methods that dictate the surface characteristics/chemistry of the material and the size of the particles [9], nonetheless the choice of model organism or cell line should also be considered. The discrepancy between the results of different studies is thus a result of testing a plethora of differently sourced materials, different surface chemistries, and concentrations [10,11]. Alginate, another example of a polysaccharide, is a ubiquitous material used in numerous medical products and applications and is a widely studied material that has demonstrated excellent properties for medical applications, i.e. biocompatibility, low toxicity, and mucoadhesion [12]. Alginate is generally regarded as safe under numerous exposure scenarios, whether oral, topical, or ophthalmic exposure [13,14] and is also an authorised food ingredient (E400-E405). When using non-toxic crosslinkers, typically Ca^{2+}, crosslinked alginate hydrogels have also been shown not to be problematic. Alginate hydrogels typically are not degradable by mammals, yet due to the physiological environment in the body, they are shown to soften and dissolve over time due to the elution and complexation of the crosslinking ions, from the hydrogel matrix [15]. Additionally, crosslinked chitosan/alginate nanoparticles with quercetin loading prepared via emulsion templating were found to have no acute toxicity and acceptable hemocompatibility [16]. Therefore, some polysaccharide particles can exhibit properties that can be of concern when their sizes, surface chemistry, and morphology are altered. Thus, when considering novel sub-micron particles and new production pathways, it is important to investigate the properties of the final material. Hence, there is a need to analyse new materials for their potentially harmful effects, in our case crosslinked alginate micro/nanoparticles, because the system used is new, and residual reagents, acetic acid, ammonia or micronized calcium carbonate, in the system could potentially cause adverse effects, even though the alginate itself, as a microcapsulation material, has been shown to be harmless [17].

In this study, crosslinked alginate particles with diameters ranging from a few hundred nanometres to several micrometres were prepared with a nano-spray dryer. Spray drying typically involves dissolving or suspending an active compound in a medium consisting of a carrier that is typically a polysaccharide matrix. While there are simple systems consisting of just a few components, typically water as solvent, a polysaccharide carrier and an active compound, there are also more complex systems that can be forced to form cross-linked systems, such as those used in this study. For example, an alginate-based system with a pH-adjustable environment to induce dissolution of an insoluble calcium source and start crosslinking of the alginate upon change of pH from alkaline to acidic [18]. While the system itself is very interesting, as it allows us to produce a crosslinked water-insoluble particle in a single manufacturing stage, it is on the other hand a complex system with a variable pH. This may not be suitable for all bioactive compounds, as they may undergo chemical changes due to changes in pH. Quercetin was used in this case a common flavonoid/polyphenol where this effect could be studied. It is also potentially problematic from a health and safety perspective due to the multiple components contained within.

Thus, in the study, we used the aforementioned alginate-based crosslinking system and adapted it for use on the Büchi B-90 nano spray dryer to produce water-insoluble sub-micron particles. The complexity of the spray drying solution, which includes alginate, acetic acid, ammonia, and micronized calcium carbonate, requires toxicological characterisation for the safety assessment, as it is no longer a simple spray-drying system consisting only of water and a soluble polysaccharide. Such small and insoluble particles are problematic as they can be inadvertently inhaled by people during the manufacturing stage, especially during the collection step where the otherwise closed system is opened and the particles are collected from the electrostatic filter. Thus, the particles were analysed morphologically, but more importantly, their potential cytotoxic and genotoxic activity was also evaluated in vitro in the human hepatocellular carcinoma cell line, HepG2, using the MTT and comet assays, respectively. In addition to these analyses, we also investigated the encapsulation efficiency and the stability of quercetin in the feed solution. Quercetin was added to the spray drying feed as a model flavonoid/polyphenol compound that is quite abundant in dietary plant sources and was as such deemed to be a good candidate to test this encapsulation system as well as to investigate the potential toxicologic effects of such particles.

2. Materials and methods

2.1. Materials

Materials for the preparation of crosslinked alginate particles: sodium alginate (low viscosity, 4–12 cP, 1 % solution in H_2O at 25 °C), glacial acetic acid, 25 % ammonia solution, calcium carbonate, pullulan, citrus pectin (26–34 % ester content), and quercetin were all supplied by Sigma-Aldrich (Darmstadt, Germany). All chemicals were of reagent grade and were used as received. For HPLC analysis quercetin (> 98 %, HPLC grade), was purchased from Sigma-Aldrich (USA), acetonitrile (≥ 99.9 %; Chromosolv; HPLC grade) was purchased from Honeywell Riedel-de Haën (USA). Absolute ethanol and formic acid (98–100 %) were purchased from Merck (Germany). Ultrapure water (18.2 MΩ cm, at 25 °C) was used to prepare all aqueous solutions.

Materials for the in vitro toxicological characterisation: Minimum Essential Medium (MEM) without phenol red. Fetal bovine serum (FBS), Na-pyruvate, Dulbecco's phosphate buffered saline (DPBS), 0.25% Trypsin-EDTA, and Triton X-100 were from Thermo Fisher Scientific (Waltham, MA, USA), ethylenediaminetetraacetic acid (EDTA), benzo[*a*]pyrene (BaP), dimethylsulphoxide (DMSO), penicillin/streptomycin, non-essential amino acids, and L-glutamine were from Sigma (St. Louis, MI, USA). The Cell Titer 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay was from Promega (Madison, WI, USA). Normal melting point agarose (NMP), and low melting point agarose (LMP) were from Invitrogen (Paisley, Scotland). All other chemical reagents were of the purest grade available and all solutions were made using Mill-Q water.

2.2. Nano-spray drying

The crosslinked alginate sub-micron particles were prepared by modifying a process described by Strobel et al. [18] developed for the encapsulation of corn oil. Briefly, the crosslinked alginate particles were prepared by spray-drying a dispersion of solubilized alginate, ammonia and acetic acid, and an insoluble source of calcium ions for crosslinking, in the form of $CaCO_3$. After the introduction of the feed into the drying chamber, the alkaline component is volatilised first as it has a significantly lower boiling point than the acetic acid. The remaining acid then reacts with the calcium source producing a soluble calcium acetate capable of crosslinking the alginate. This procedure was adapted for nano-spray drying, where certain limitations with the processing equipment needed to be considered. We used the largest spray mesh with 7 μm perforations. Thus, reducing the particle size of the insoluble particles was required for the material to pass easily through the spray mesh. Therefore, calcium carbonate was milled in a planetary ball mill

Table 1
Composition of dispersions used in spray drying on the Nano Spray Dryer.

Reagent	xA	xAPe	xAPu
Sodium alginate	100 mg	75 mg	75 mg
Pectin	/	25 mg	/
Pullulan	/	/	25 mg
Calcium carbonate	1 mmol	1 mmol	1 mmol
Aqueous ammonia (25 %)	10 mmol	10 mmol	10 mmol
Glacial acetic acid	5 mmol	5 mmol	5 mmol
Deionized water	*ad* 100 mL	*ad* 100 mL	*ad* 100 mL

Non-crosslinked alginate was also prepared by nano spray drying where the feed solution was a 0.1 % solution of polysaccharide in water.

in absolute ethanol. Seven grams of calcium carbonate was suspended in 25 mL of absolute ethanol and the suspension was milled in a 100 mL alumina ceramic lined milling jar with ~50 mL of 10 mm alumina ceramic milling media. The material was milled in 4 cycles of 50 min each, with 10 min between each cycle to allow for cooling of the milling jar. The temperature during milling did not exceed 60 °C. The final size of the calcium carbonate particles was below one micron. The feed solution was prepared by first dissolving the polysaccharide(s) in the appropriate amount of deionized water, followed by the addition of calcium carbonate. Calcium carbonate was dispersed using a 3 mm ultrasonic probe which has an amplitude of 170 μm and an oscillating frequency of 20 kHz. The dispersion was sonicated for 60 s, which was enough to yield a homogeneous dispersion of calcium carbonate. Following the dispersion of calcium carbonate, the pH was adjusted by first adding the ammonia solution, followed by slow addition of acetic acid under vigorous stirring. The final pH of the feed solution was around 10. Where quercetin was needed it was added as a fine powder which was quickly dissolved (within 30 s). The feed solution was then immediately taken to the spray drier and the process started. The spraying solution contained a total of 0.1 % polysaccharide carrier, as higher viscosities inhibited proper spray formation on the piezoelectric nozzle. The material was recovered on an electrostatic filter.

Spray-drying conditions were as follows; an inlet temperature of 120 °C and an outlet temperature, depending on the system, between 40 and 43 °C. The spraying rate was around 60 mL per hour. Additional parameters were as follows, a spraying frequency of 125 kHz, flow rate across the spraying mesh was 100 %, spraying intensity was 80 % and the drying gas flow rate was 100 L/min. The spray feed compositions are described in Table 1. A two-fold excess of ammonia over acetic acid was used, and a five-fold excess of acetic acid to calcium carbonate was used to guarantee proper conditions for dissolution. The feed included alginate to produce crosslinked alginate beads (xA), or admixed polysaccharides where 25 % of alginate was replaced by either pectin (xAPe) or pullulan (xAPu). Replacing 25 % of the alginate with either pullulan or pectin was made in an attempt to improve the spraying characteristics of the feed solution as pure alginate solutions are difficult to atomize on the nano spray drier, even at such low concentrations (0.1 %). The presence of an asymmetrical cone was observed as the main issue which we wanted to improve. Quercetin was added to these formulations at a loading of 20 % of the total polysaccharide content.

2.3. Total encapsulation efficiency determination by HPLC

To a certain mass of microcapsules, absolute ethanol was added, so that the final mixture theoretically corresponds to 1 mg/mL of active substance. The dispersion was vortexed to disperse the capsules followed by two 10-min sonication cycles to extract the quercetin from the capsules. The dispersions were then centrifuged and the supernatant was taken for HPLC analysis. 20 μL sample was applied on C18 column (Zorbax Eclipse Plus; 4.6 × 150 mm, I.D. 3.5 μm; Agilent Technologies) coupled with a C18 pre-column (Eclipse XBD; 4.6 × 12.5 mm, I.D. 5 μm; Agilent Technologies) and analysed on HPLC Infinity 1260 system (Agilent Technologies) with following gradient mode: from 0 to 10 min mobile phase A (0,1 % formic acid) was decreased from 75 % to 70 % A, from 10 min to 20 min mobile phase A was further decreased to 35 % A and in 1 min increased to 100 % of mobile phase B (acetonitrile with 0,1 % formic acid) and finally 5 min decreasing to starting 75 % A. The flow rate was set to 0,8 mL/min, and column temperature was set to 35 °C. Quercetin elution was monitored at 370 nm and identified with recorded DAD spectra in the range from 200 nm to 560 nm and retention time in comparison with external quercetin standard solutions. Quercetin quantity was determined with external standards of quercetin solutions in the linear range from 0.45 μg/mL to 11 μg/mL and regression factor, R^2, 0,997. LOD and LOQ were calculated with Data analysis tool in Excel software according to ICH guidelines and were 0.64 μg/mL and 1.93 μg/mL respectively.

2.4. Scanning electron microscopy

Morphological observations of the obtained materials were performed with SEM (Quanta 250; FEI Company, USA) under high vacuum, at an acceleration voltage of 10 kV, with a spot size of 2.0, approximately at 9 mm working distance. Prior to imaging, the dry samples were mounted on aluminium stubs with carbon tape and sputtered with gold in an argon atmosphere ($I = 15$ mA, $t = 240$ s; Q150R ES Sputter and Carbon Coater; Quorum Technologies Ltd.). The particle sizes were determined from the micrographs obtained, using the FIJI image processing package (ImageJ software; FIJI). Two hundred particles were analysed for each sample.

2.5. Laser light scattering (particle size and zeta potential)

The hydrodynamic particle size and zeta potential of xAQ, xAPeQ and xAPuQ were measured before cytotoxicity and genotoxicity testing. The measurements were performed over a period of 24 h. Immediately upon dispersion, after 2 h and finally, after 24 h, in order to examine potential particle aggregation. The measurements were performed in MilliQ water and the growth medium for the HepG2 cell line, which consisted of 1 mL 100 mM Na-pyruvate, 1 mL 1 % penicillin/streptomycin solution, 1 mL 1 % L-glutamine, 10 mL FBS and 86.54 mL MEM.

2.6. Evaluation of the cytotoxic and genotoxic activities

The cytotoxic and genotoxic activities were evaluated for the following materials: i) Non-crosslinked alginate with calcium carbonate, sample designation AC; ii) Crosslinked alginate with added pectin (ratio 3:1) sample designation xAPe; iii) Crosslinked alginate with added pullulan (ratio 3:1), sample designation xAPu; iv) Crosslinked alginate with quercetin (20 % loading), sample designation xAQ; v) Crosslinked alginate with added pectin and quercetin (20 % loading), sample designation xAPeQ; and vi) Crosslinked alginate with added pullulan and quercetin (20 % loading), sample designation xAPuQ.

2.7. Cultivation of HepG2 cells

HepG2 cells were acquired from Cell bank – ATCC (American Type Culture Collection; HB-8065™), and were grown at 37 °C and 5 % CO_2 in Minimum Essential Medium (MEM) without phenol red supplemented with 10 % FBS, 100 IU/mL penicillin/streptomycin, 1 % non-essential amino acids, 0.1 g/mL Na-pyruvate, and 2 mM L-glutamine.

2.8. Cytotoxicity - MTS assay

The cytotoxicity of nano-spray dried alginate particles was determined with Cell Titer 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay according to the manufacturer's instructions, with minor modifications. The HepG2 cells were seeded onto a 96-well microplate (Nunc, Thermo Fisher Scientific, USA) at a density of 8.000 cells/well and incubated for 24 h at 37 °C in 5 % CO_2 to attach. The growth medium

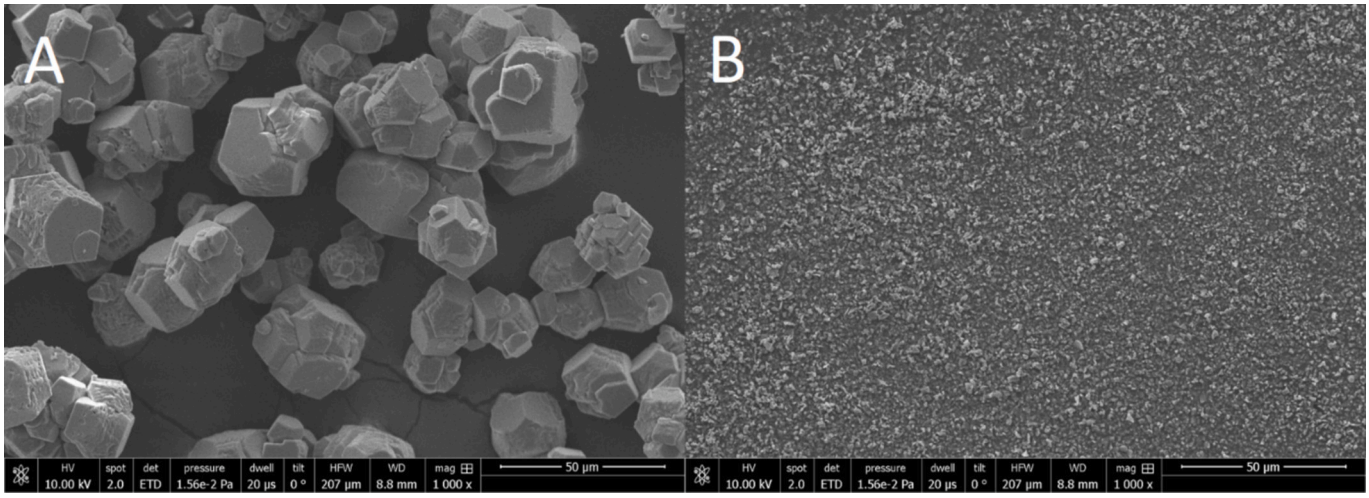

Fig. 1. Comparison of calcium carbonate before (A) and after (B) milling. 1.000× magnification for both samples. The scale bar is 50 µm.

was then replaced with fresh medium containing 0, 1, 5, 10, 50, 100, and 500 µg/mL nano-spray dried alginate particles. After the 24-h exposure freshly prepared mixture of MTS:PMS solution (20:1) was added to each well and incubated for an additional 3 h. Subsequently, the cell viability was measured using the spectrofluorimeter (Synergy MX, BioTek, Winooski, VT, USA) at 490 nm. Statistical analysis was performed using one-way analysis of variance (1-way ANOVA), and Dunnett's comparison test, $p < 0.05$ was considered significant. Three independent experiments were performed each time in 5 replicates where each replicate represented one well, respectively.

2.9. Genotoxicty - Comet assay

The potential induction of DNA strand breaks in HepG2 cells after the exposure to nano-spray dried alginate particles was assessed by single cell gel electrophoresis (SCGE) also known as the comet assay. HepG2 cells were seeded at a density of 80.000 cells/well into 12-well microtiter plates (Corning Costar Corporation, Corning, NY, USA) and incubated for 24 h at 37 °C and 5 % CO_2. After that, the growth medium was replaced with fresh medium containing 0, 10, 50, and 100 µg/mL nano-spray dried alginate particles and incubated for 4 and 24 h. In each experiment, benzo[*a*]pyrene (BaP; 30 µM for 24-h exposure and 50 µM for 4-h exposure) was included as a positive control. At the end of exposure, the cells were harvested by trypsinization, and DNA damage was determined as described by Singh et al. (Singh et al., 1988) with minor modifications by Bittner et al. (Bittner et al., 2021). Images of at least 50 randomly selected nuclei per experimental point were analysed using a fluorescence microscope (Eclipse 800, Nikon, Japan) and the image analysis software Comet IV (Perceptive Instruments, UK). Three independent experiments were performed for each treatment condition. The results are expressed as % of tail DNA. The differences in the % of tail DNA between treatments and control within each experiment were analysed using the Kruskal-Wallis one-way analysis of variance and Dunn's comparison test in GraphPad Prism V9 (GraphPad Software, San Diego, CA, USA), where $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Crosslinking process for nano-spray dried alginate particles

The nano-spray dryer has certain specifics when it comes to the material that can be produced on the machine. We are severely limited by the spraying mechanism, which is based on a piezoelectric membrane of defined porosity. So, the material passing through the perforated

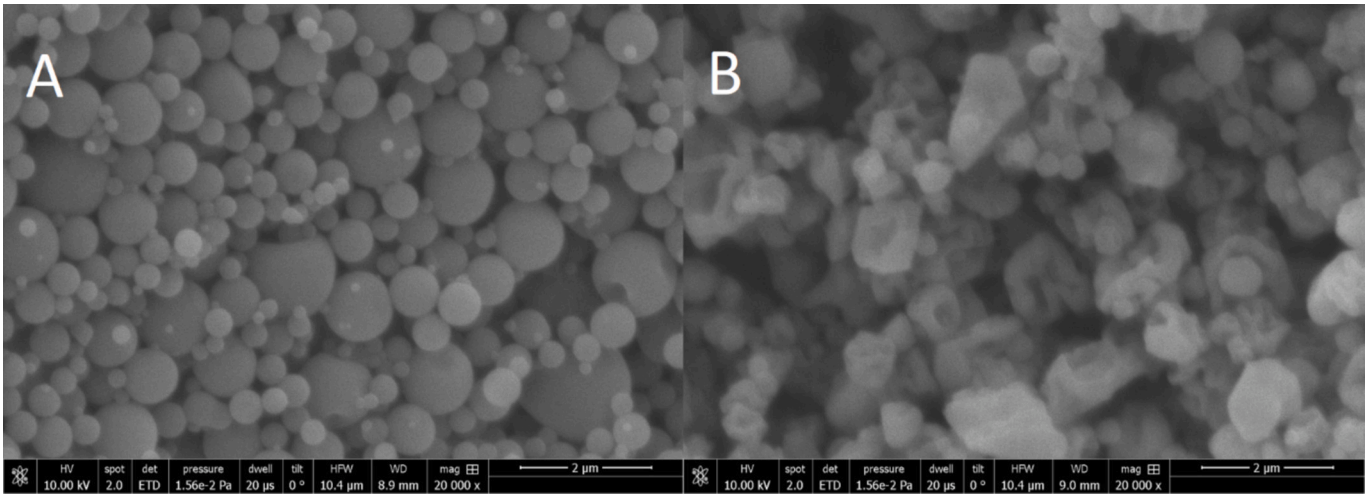

Fig. 2. SEM Image of pure nano-spray-dried alginate particles (A), and crosslinked alginate particles (B). The pure alginate particles have a smooth surface with a uniform round morphology. Crosslinked alginate on the other hand has an irregular morphology. The presence of undissolved or unreacted calcium carbonate is evident at 20.000× magnification, scale bar is 2 µm.

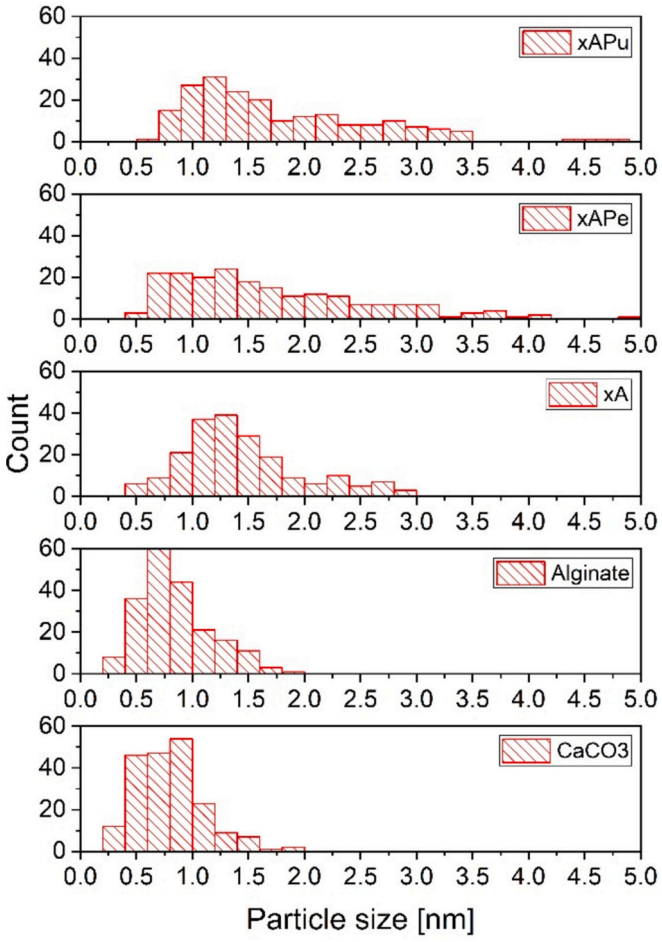

Fig. 3. Particle size distribution of different materials. xAPu denominates crosslinked alginate where 25 % of the alginate was replaced with pullulan, similarly xAPe denominated crosslinked alginate where 25 % of the alginate was replaced by pectin. xA denominates crosslinked alginate, Alginate is pure nano spray dried alginate and CaCO3 is the ball milled calcium carbonate that was used as the calcium source for the crosslinking process. Detailed compositions are described in Table 1.

mesh needs to be substantially smaller in size than the aperture in the membrane, which in our case was 7 μm. The insoluble material, the calcium carbonate thus had to be micronized to a sufficient degree that would allow it to pass through the membrane unobstructed and also prevent potential clogging of the membrane, which would have severely reduced spraying efficiency and some degree of particle size control.

The calcium carbonate was thus ball milled in a non-aqueous medium (absolute ethanol). The total effective milling time was 200 min, which was done in 4 cycles of 50 min each. After each cycle a 10-min pause was used to allow for cooling of the milling jar. Seven grams of calcium carbonate was dispersed in 25 mL of absolute ethanol and milled with alumina medium.

The resulting calcium carbonate dispersion was dried under reduced pressure and analysed by SEM (Fig. 1). It was determined that the particles had a mean size of roughly 0.8 μm, which was sufficient for our applications. For use, the particles were dispersed in the carrier solution and sonicated for 60 s to achieve homogenous dispersion.

The crosslinking process is a delicate balance of pH control before and during the spray drying process. The pH needs to be alkaline in order for the calcium carbonate particles to remain effectively suspended in the medium. During the spray drying process, the base needs to be effectively removed from the droplets for the pH to decrease where calcium carbonate solubilization can occur. Several formulae were tested, ranging from solid organic acids like EDTA, citric, adipic, and succinic acid, to glucono-delta-lactone and acetic acid. All, but acetic acid, were found to either be problematic as they acted too slow to induce crosslinking or dissolution of calcium carbonate. Thus, acetic acid was chosen. In the end, the system is similar to that presented by Strobel et al. [18] was chosen. Based on ammonia, acetic acid, sodium alginate, and micronized calcium carbonate, the particles were prepared on a nano-spray dryer. The mixing sequence of the reactants was crucial, mainly the acetic acid had to be added after the ammonia solution, to avoid solubilization of calcium carbonate and inducing the crosslinking. The pH of the final dispersion was around 10.

The spray dried materials were analysed by SEM. Pure alginate had a uniform particle morphology, primarily in the form of smooth round particles. For the crosslinked alginate sample, a uniform particle distribution was observed, but there was a large amount of calcium carbonate particles, which were visible as irregularly shaped particles with sharp corners (Fig. 2). The presence of these particles suggests incomplete dissolution. If required, dissolution could be improved by decreasing the amount of CaCO3 in the feed (tuning the reagent ratio). The temperature, spray path, and residence time of the particles in the drying chamber are also important parameters, which could be further researched in the future.

The particle size distribution was evaluated from SEM images by taking 200 random particles and measuring them manually. As a rule, for the irregularly shaped particles, their largest lateral dimension was considered. The results are summarized in Fig. 3. The calcium carbonate was effectively milled to yield a powder with a mean particle size of 0.80 (+/− 0.30) μm.

The nano spray-dryer can produce particles in the sub-micron range, however, the efficiency is low, and it rarely produces materials that could be considered as true nanomaterials (i.e. at least one dimension at 100 nm or smaller). The efficiency also strongly depends on the composition and constituents, as well as the chosen spraying mesh. In our case, the spray mesh was the largest available with 7 μm perforations to facilitate the passage of micronized calcium carbonate. The smaller 4 μm mesh, which would reduce the particle size of the produced powders,

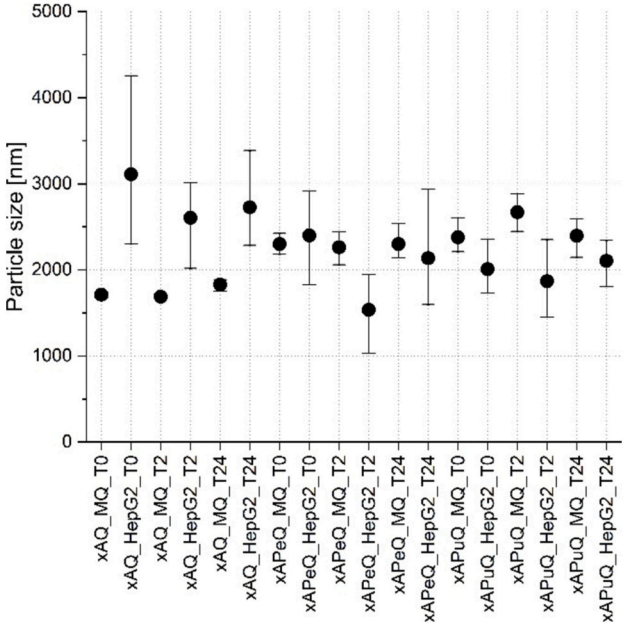

Fig. 4. Particle size measurements by dynamic light scattering at time zero (T0), after 2 h (T2), and after 24 h (T24) for different types of nano spray-dried materials in MilliQ (MQ) and HepG2 cultivation medium (HepG2). Sample identifiers; xAQ – Crosslinked alginate with quercetin, XAPeQ – Crosslinked alginate/pectin with quercetin, XAPuQ – Crosslinked alginate/pullulan with quercetin.

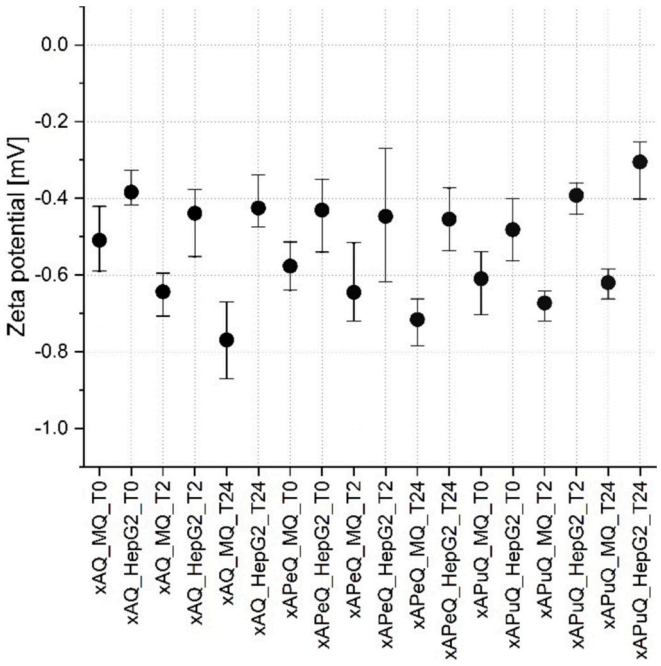

Fig. 5. Zeta potential measurements by electrophoretic mobility at time zero (T0), after 2 h (T2), and after 24 h (T24) for different types of nano spray dried materials in MilliQ (MQ) and HepG2 cultivation medium (HepG2). Sample identifiers; xAQ – Crosslinked alginate with quercetin, XAPeQ – Crosslinked alginate/pectin with quercetin, XAPuQ – Crosslinked alginate/pullulan with quercetin.

could not be chosen due to clogging issues. For the pure alginate, roughly 75 % of the particles, that were measured using SEM, could be classified as being below the size of 1 μm. Crosslinked alginate on the other hand had around 25–30 % of the particles below 1 μm. The influence of added pullulan and pectin on the particle sizes was negative (Fig. 3). While the spraying characteristics were improved, i.e. spray cone formation, the particle sizes were larger on average and a smaller percentage of particles were below the 1 μm range in the mixed Pullulan/Alginate system, around 10 % of all analysed particles were below 1 μm. The combined Pectin/Alginate system had a similar particle distribution below 1 μm as the crosslinked alginate, around 25 %. The particle size range was generally larger, although the number of larger particles approaching 4–5 μm, was low. Overall, pure nano spray dried alginate had a mean particle size of 0.83 (+/− 0.32) μm, while the crosslinked alginate had a mean particle size of 1.43 (+/− 0.54) μm. Replacing 25 % of the alginate with either pectin or pullulan resulted in mean particle sizes of 1.70 (+/− 0.87) and 1.75 (+/− 0.80) μm, respectively.

3.2. Particle size and zeta potential of nano-spray dried particles with added quercetin

Particle size distribution and zeta potential for the quercetin-loaded materials were measured in MilliQ water and growth medium for the HepG2 cell line. The results are presented in Figs. 4 and 5. Based on the data provided by SEM imaging and DLS, we can say that regardless of medium, either MilliQ water or growth medium for the HepG2 cell line, the particles did not aggregate over time, as particle sizes observed on SEM and by DLS appeared to be in a similar range. Except in the case of pure crosslinked alginate, where a statistically significant increase in particle size was observed by DLS over a 24-h period as a function of the dispersion medium, as the HepG2 medium seemed to cause some aggregation in the xAQ samples. Differences between other samples show no statistical significance, hence we were able to exclude any effect of medium on sample aggregation. Thus, it appears that the inclusion of pectin or pullulan reduces the potential for aggregation of these materials. Full data of Tukey's HSD analysis can be found in supplementary info.

The particles exhibited a near-neutral zeta potential; thus, their dispersions did not appear to be colloidally stable. Furthermore, they did not exhibit a preferential surface charge. As alginate was used in its sodium salt, which has a negative surface charge, it can be safely assumed that the majority of the deprotonated carboxylic groups have been effectively crosslinked via salt bridging with bivalent calcium cations, stemming from in-situ solubilized calcium carbonate. Regardless of the data and the small differences, we have performed a statistical analysis which has detected statistically significant differences between the samples, however, due to the sensitivity of the machine, any presence of a dominating surface charge would be detected as a sharp increase in absolute zeta potential value. Due to the nature of the samples and the measured values being between 0 and − 1 mV, the differences, while statistically significant, do not represent any substantial difference in surface charge. The statistically significant differences can be explained by a small measurement error.

3.3. Encapsulation efficiency of quercetin in the nano spray-dried particles

Quercetin exhibits increased solubility in alkaline solution due to (poly)phenate formation [19]. However, the stability of quercetin in an alkaline solution is also severely diminished. The time from preparing the solution up to spray-drying was initially deemed short enough to preserve the quercetin in its native form. However as the pH of the spraying solution was ~10, where the degradation of quercetin was extreme, and in conjunction with the high temperatures [20] and low spraying efficiency of the nano-spray dryer we have found that most of the quercetin in its native form was lost in the spray-drying process.

The theoretical amount of quercetin in the powders would be 1 mg of quercetin per 9 mg of powder, assuming 100 % encapsulation efficiency. Our analysis indicated that we were able to encapsulate 2.29 (+/− 0.07) μg, 8.58 (+/− 0.16) μg, and 20.59 (+/− 0.23) μg in a 9 mg sample of powder of Quercetin in xAQ, xAPeQ, and xAPuQ, respectively. This represents 0.23 %, 0.86 %, and 2.06 % encapsulation efficiency, respectively.

These results indicate that the tested system is not suitable for the encapsulation of Quercetin, which is corroborated by stability studies of Quercetin in alkaline solution recently published by our group [20]. Even at pH levels only slightly above neutral, the degradation of quercetin increases dramatically. Even though the encapsulation efficiencies are extremely low, we can observe some protective effects of both pectin and pullulan, i.e. an increase in the total quercetin content over 375 % and 900 %, in the Alginate/pectin and Alginate/pullulan capsules. However, the absolute levels of intact quercetin in the capsules are still extremely low. Nevertheless, it is important to note that while quercetin is only present in small amounts in the produced particles, the degradation products of quercetin are likely also present. Such degradation products were identified in a previously published study by our group [20]. As such it makes sense to also evaluate the toxicologal profile of particles with quercetin and its degradation products.

3.4. Cytotoxicity and genotoxicity assessment of crosslinked particles

Toxicity, in particular genotoxicity, is a key aspect in assessing the safety of bioactive compounds intended for various human applications. In the present study, the cytotoxicity and genotoxicity of alginate-based nano-spray dried materials were evaluated in vitro in the metabolically competent HepG2 cell line, derived from human hepatocellular carcinoma. HepG2 cell line is one of the most widely used human cell models for drug metabolism and hepatotoxicity studies [21–23]. They are important in toxicity testing because the liver is the main detoxifying

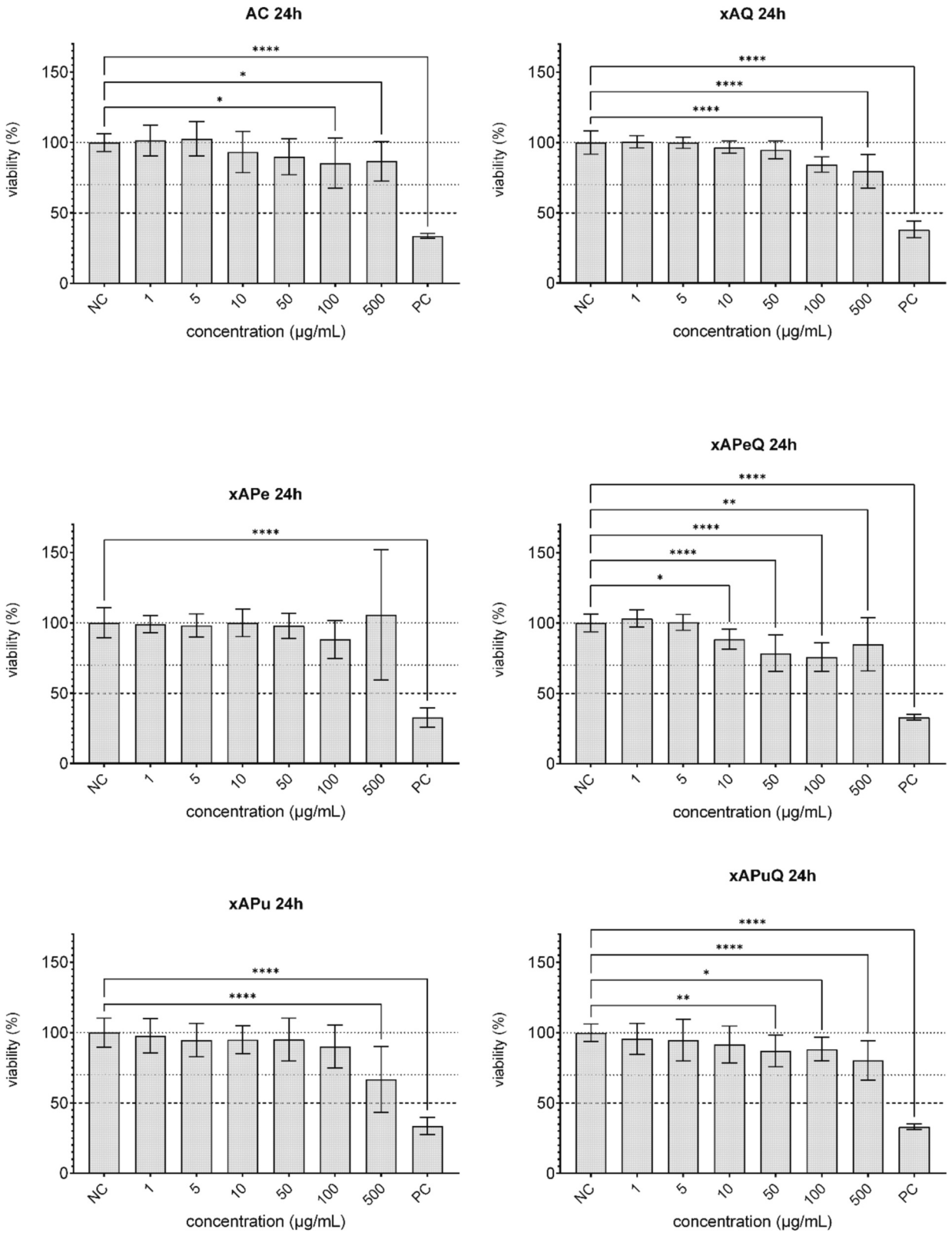

Fig. 6. HepG2 cell viability after 24 h of exposure to AC, xAPe, xAPu, xAQ, xAPeQ, and xAPuQ nano-spray dried materials accessed by MTS assay. DMSO (3 %) served as a positive control (PC). Results are presented as % of viable cells ± SD normalized to negative control (NC). Statistical analysis was performed by one-way ANOVA with Dunnett's post hoc test; [$p < 0.05$ (*), p < 0,01, $p < 0.0001$ (****)].

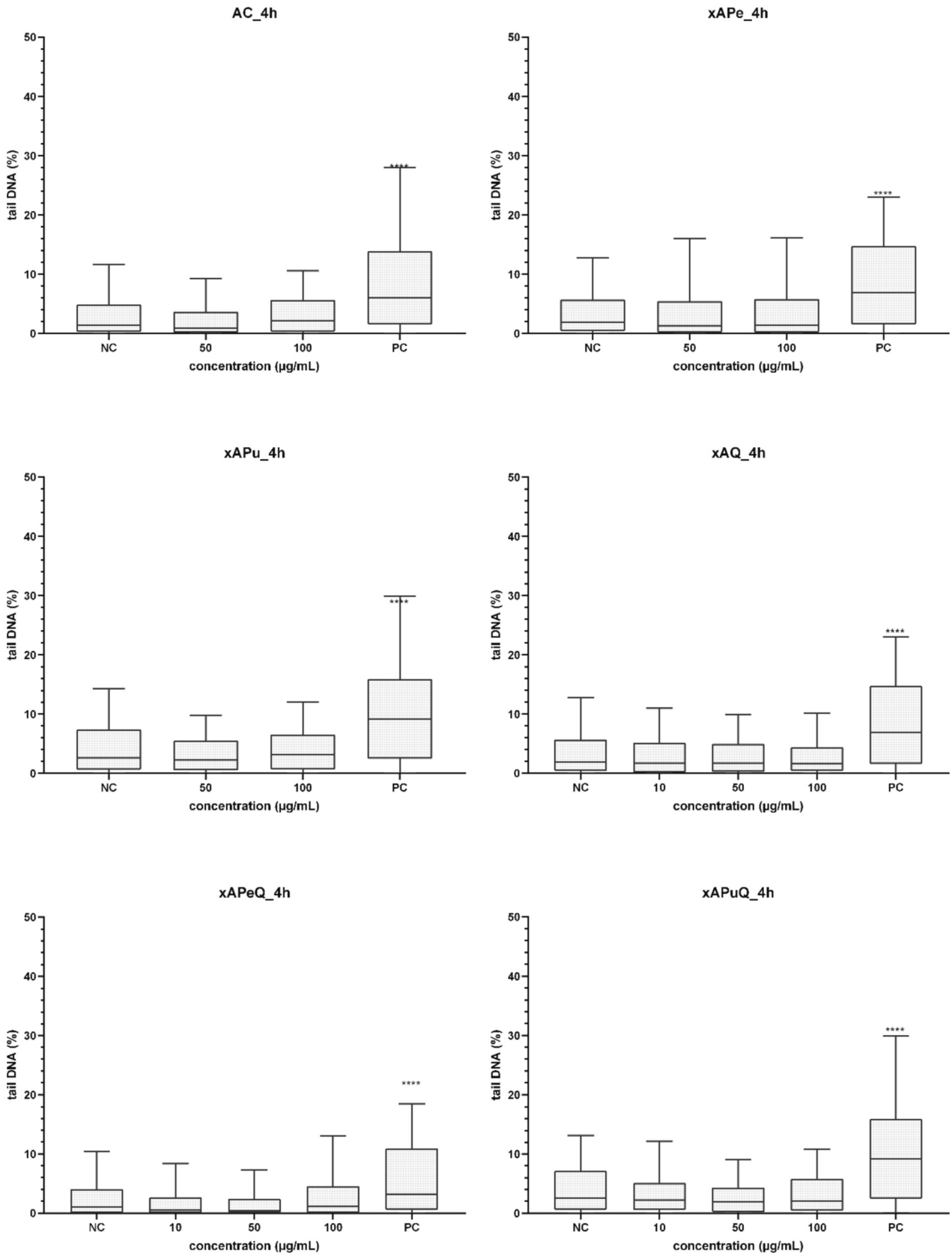

Fig. 7. DNA strand breaks in HepG2 cells were assessed by the comet assay after 4 h of exposure to nano-spray dried materials. Benzo[*a*]pyrene (50 μM) was considered as a positive control (PC). Fifty-five nuclei were measured per experimental point and presented in box plots using GraphPad Prism software. Statistical analysis was performed by Kruskal-Wallis test [$p < 0.05$ (*), $p < 0.00001$ (****)].

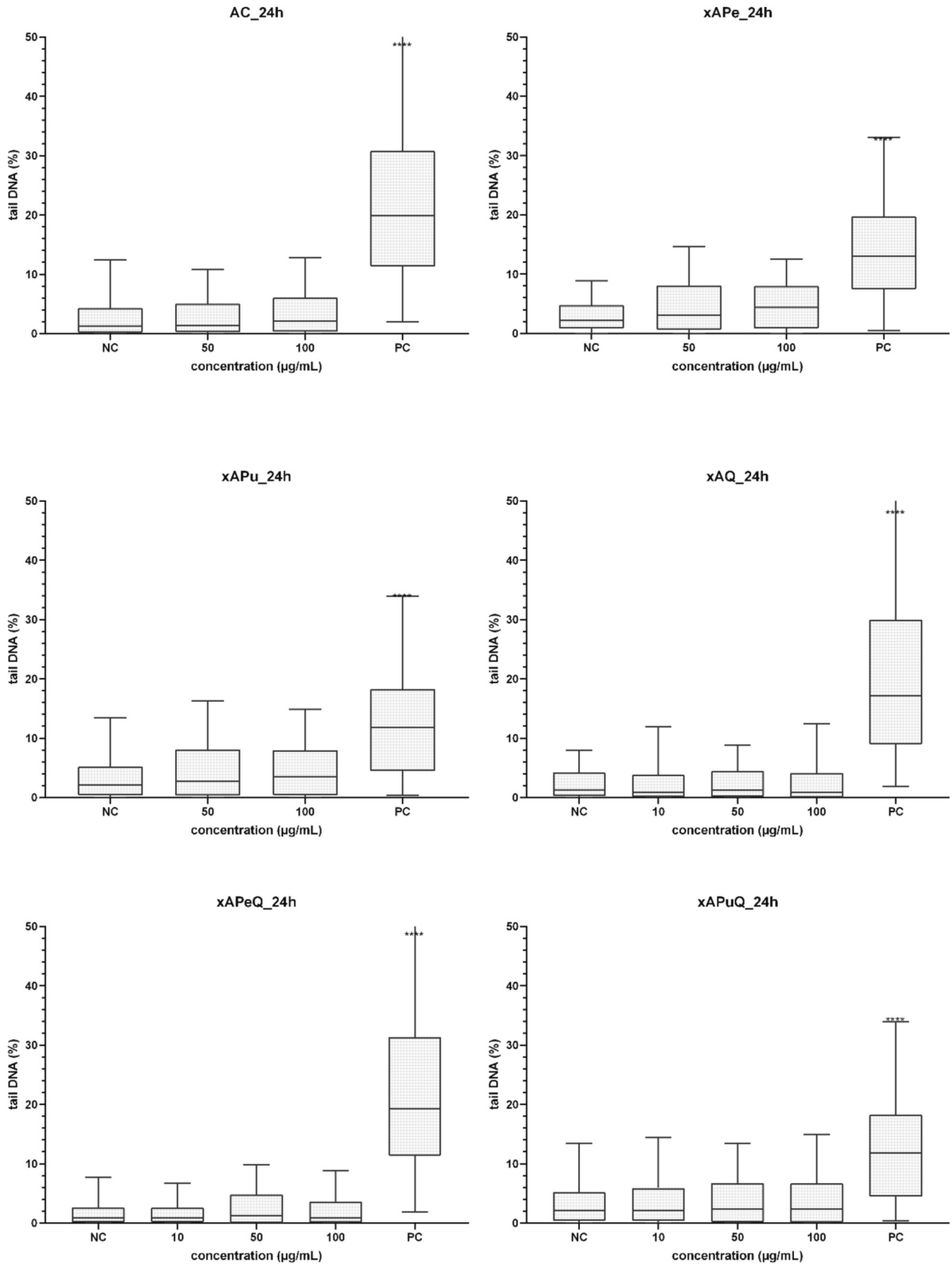

Fig. 8. DNA strand breaks in HepG2 cells were assessed by the comet assay after 24 h of exposure to nano spray-dried materials. Benzo[*a*]pyrene (50 μM) was considered as a positive control (PC). Fifty-five nuclei were measured per experimental point and presented in box plots using GraphPad Prism software. Statistical analysis was performed by Kruskal-Wallis test [$p < 0.05$ (*), $p < 0.00001$ (****)].

organ in the human body and is mainly responsible for drug metabolism and drug-drug interactions [23]. Furthermore, HepG2 cells have an unlimited lifespan and stable phenotype and are characterized by high availability and easy handling [22]. The cytotoxicity of nano-sized materials is an important parameter that should be evaluated when developing a drug delivery system, especially as assessing cell viability prior to genotoxicity testing can exclude false-positive results due to DNA fragmentation, which can occur during cell death [24]. The impact of nano-spray dried materials on the viability of HepG2 cells after 24-h exposure was determined with the MTS assay, a simple, accurate, and quantitative colorimetric method for determining the proliferation, viability, and cytotoxicity of both suspended and adherent cells, where the amount of formed formazan crystals colorimetrically detected (490 nm) is directly proportional to the number of live, metabolically active cells in the culture [25,26]. The results (Fig. 6) showed that the studied nano-spray dried materials did not decrease cell viability by >25–30 % at the concentrations applied (up to 500 μg/mL), except from xAPu. Cells exposed to xAPu nano-spray dried materials showed a significant decrease in cell viability of approximately 43 % compared to the negative control at the highest concentration (Fig. 8). The positive control DMSO (3 %) reduced HepG2 cell viability by approximately 44 % after 24 h, compared to the negative control.

There is limited literature data on the toxicity and genotoxicity of nano-sized alginate in vitro, nevertheless most in vitro or in vivo studies conducted on macro-, micro-, or nano-sized alginate report good biocompatibility, inertness, and biodegradability [27–33]. Furthermore, the use of alginate is approved by the Food and Drug Administration (FDA) [34]. Although the literature data on nano-sized material-induced genotoxicity has increased in the last years, the results remain inconsistent, inconclusive, or even contradictory [24]. In the present study, DNA damage induced by alginate-based nano-spray dried materials after 4 and 24-h exposure was assessed by the comet assay, a simple, adaptable, versatile, and fast quantitative in vitro method to measure DNA damage and repair at the individual cell level [35]. The method enables the detection of single- and double-strand DNA breaks (strand breaks and incomplete excision repair sites), alkali-labile sites, DNA-DNA, and DNA-protein cross-linking [35]. Our results show that alginate-based nano-spray dried materials did not induce DNA damage after 4 and 24 h (Figs. 7 and 8) of exposure, at concentrations up to 100 μg/mL. The positive control benzo[*a*]pyrene (50 μM for 4-h and 30 μM for 24-h exposure) induced statistically significant DNA damage at both exposure times, as expected. There are no studies available in the literature addressing the (geno)toxicity of nano-sized polysaccharides (alginate, pectin, or polulan) investigated as components of the nano-spray-dried materials in this study. Only two studies have been published investigating the mutagenicity of macro-sized pectin [36] and pullulan [37] using the Ames assay, both of which reported negative results.

4. Summary

This study focuses on the properties of crosslinked alginate capsules prepared on a nano-spray dryer. The size of the particles ranged from a few hundred nanometres to several microns, and thus their properties are largely unknown from a health perspective. Handling of such powders, which can linger in the air for long periods, is typically classified as being hazardous, and small particles, for example under 2.5 μm are capable of penetrating deeper into the lung tissue should they be advertently or inadvertently inhaled. To summarize, we were able to prepare crosslinked alginate and mixed polysaccharide particles on a nano-spray dryer by successfully adapting a system intended to produce larger particles on a more robust mini-spray dryer. The particles had a rough and uneven morphology, indicating only partial dissolution of the calcium carbonate component. Furthermore, the particles did not appear to aggregate in milliQ water or HepG2 cell growth medium. They had a negligible surface charge, which indicated successful crosslinking and cationic complexation with Ca^{2+} ions as sodium alginate has a strongly negative surface charge due to deprotonated carboxylic groups.

Quercetin was included in the capsules as a model flavonoid/polyphenol compound. As was seen, during material preparation, quercetin shows extremely poor stability in the spraying solution/dispersion due to the alkaline nature of the solution. The rate of degradation of quercetin in even mildly alkaline solution is rapid. In our case, the pH of spraying dispersion was around 10. The total encapsulation efficiency of quercetin in the capsules was thus very low, from 0.23 to 2.06 %, with some protective effect observed by the addition of pectin and pullulan. Regardless of this extremely low encapsulation efficiency, the particles were analysed for their cytotoxicity and genotoxicity as quercetin degradation results in numerous compounds.

Tests showed that only mixed Alginate/Pullulan particles were cytotoxic at the highest tested concentration (500 μg/mL) where a 43 % reduction in cell viability was observed in the MTS test. Loss of cell viability in the other samples did not exceed 25–30 % and are not considered cytotoxic. Genotoxicity as established by the comet assay showed that our materials were not genotoxic at tested concentrations up to 100 μg/mL.

Overall the study provides some interesting insights into materials produced by the nano-spray drying system. While the possibility of producing powders ranging from small micrometre-sized particles down to the scale of several hundred nanometres certainly exists and is tempting, the processing efficiency is a major hindrance. Not only does the encapsulation system need to be tailored specifically for the nano spray dryer (i.e. micronization of the calcium source), but the pH also needs to be adjusted accordingly severely limiting the usefulness of such a system with molecules that show reduced stability at alkaline or acidic pH. A concern also exists when handling such materials as they tend to linger in the air and due to their size are a potential hazard. This is what prompted us to conduct this study in the first place, especially due to the complexity of the spraying system. Overall the usefulness of the nano-spray drying system is limited and is probably better suited for less complex spray-drying applications, producing relatively well-defined powders from clear solutions as opposed to dispersions. In this way, spraying efficiency would not suffer and production costs could be kept down. Regardless of process efficiency, we have demonstrated that the crosslinked alginate particles could be produced on the nano-spray dryer and that they were not cytotoxic and did not show signs of genotoxicity under the tested conditions in the HepG2 cell line. On the other hand, we also demonstrated that the system employed was not suitable for the encapsulation of quercetin, as extensive degradation of the molecule was observed and the encapsulation efficiency was practically zero.

CRediT authorship contribution statement

Jaka Levanič: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Ilja Gasan Osojnik Črnivec:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Iza Rozman:** Writing – original draft, Methodology, Investigation, Formal analysis. **Mihaela Skrt:** Writing – original draft, Methodology, Investigation, Formal analysis. **Alja Štern:** Writing – review & editing, Writing – original draft, Supervision. **Bojana Žegura:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Nataša Poklar Ulrih:** Writing – review & editing, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.137750.

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