**Influence of surface morphology and structure of alginate microparticles on the bioactive agents release behavior**

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**ABSTRACT**

The structure-property relationship in alginate microparticles (microspheres and microcapsules) prepared with or without *Trichoderma viride* spores (*Tv*) was investigated. Surface morphology, structure and release behavior from alginate microparticles strongly depend on calcium concentration and presence of *Tv* and chitosan layer. All microparticles exhibited a granular surface structure with substructures consisting of abundant smaller particles. *In vitro* active agents release study revealed that the increase in calcium cation concentration as well the presence of the chitosan layer reduced the release rate of both, *Tv* and calcium cations from alginate microparticles. The underlying *Tv* release mechanism from microspheres is anomalous transport kinetics, whereas from microcapsules is controlled by Type II transport. The transition of glassy structure to rubbery state is slower on microcapsule than on microsphere surface due to bigger surface grains. The differences in microparticle surface properties did not affect the mechanism controlling calcium ions release detected as diffusion through microparticles.

**Keywords**: alginate microparticles, biofertilizer, release mechanism, structure/property relationship, *Trichoderma viride*

**1. Introduction**

The use of agrochemicals in agriculture had substantial repercussions for the environment, food security as well as human health because some of them are persistent organic pollutants. To diminish the overall exposure to agrochemicals, the worldwide intention is the restriction of their use and application of environmentally friendly formulations like biofertilizers and/or biopesticides. A biofertilizer is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). Biofertilizer formulations usually contain a living microorganism (bacteria, fungi, nematodes) and a suitable carrier together with additives. Efficient formulation demands a carrier material which must preserve or maintain living organisms in a viable condition during storage and transport as well as must keep its functional properties after application. Current biofertilizer formulations for agriculture are available as powder, dust, granule, oil-emulsion or microcapsules. Microencapsulation is an advanced technology superior to prepare biofertilizer agroformulations in terms of protection living organism, improvement of their viability and possibility of controlled release into the field (John, Tyagi, Brar, Surampalli, & Prévost, 2011).

Recently, we have shown the possibility of simultaneous encapsulation of copper (Vinceković et al., 2016, 2017; Vinceković, Jurić, Đermić, & Topolovec-Pintarić, 2017) or calcium cations(Jurić, Đermić, Topolovec-Pintarić, & Vinceković, 2019) with *Trichoderma viride* spores (*Tv*) in alginate microparticles. *Trichoderma* species are among the most prevalent culturable fungi in soils which antagonistic activity against plant pathogens and promotion of soil fertility make them as valuable biofertilizer. Knowledge of mechanism involved in the release of *Tv* from microparticles is important for the development of agroformulations based on biopolymeric materials.

The underlying hypothesis of our work is that improved understanding of the structure-property relationship in a biopolymer based agroformulation enhances the ability to control the release properties of a biofertilizer and may aid in developing microparticles with specifically tailored properties. The aim of the present work was to investigate the effect of calcium cation concentration, and the presence of *Tv* and chitosan layer on the structural properties of alginate microparticles and the release behavior with the intention of delivering bioactive agents to plants at the rate that closely approximates plant demands over an extended period.

**2. Materials and methods**

*2.1. Materials*

Alginic acid sodium salt, from brown algae, (CAS Number: 9005-38-3, M/G ratio of ∼1.56, molecular weight 280 000) g mol-1 was purchased from Sigma Aldrich (USA). Medium molecular weight chitosan (CS) (CAS Number: 9012-76-4, 190,000-310,000 Da, Degree of deacetylation: Min 75%) was obtained from Across Organic (USA). A commercially available product CaCl2 was purchased from Kemika (Croatia) and fluorescence dye, eosin, form Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and used as received without further purification.

An indigenous isolate of *Trichoderma viride* originated from parasitized sclerotia of *Sclerotinia sclerotiorum* was used in all experiments (Topolovec-Pintarić, Žutić, & Đermić, 2013). To obtain spore suspensions, the *Trichoderma viride* was grown in Erlenmeyer flasks containing potato dextrose broth (Vinceković et al., 2016).

**2.2. Microparticles preparation**

Both, microspheres (MS) and microcapsules (MC) are microparticles. A microsphere is a solid matrix particle, whereas a microcapsule has an inner core and outer shell. We have prepared MS in one step by ionic gelation, whereas MC was prepared in two steps by ionic gelation and polyelectrolyte complexation (Vinceković et al., 2016). Ionic gelation involves the preparation of MS by dripping a solution of sodium alginate without or with *Tv* into calcium chloride solution using Encapsulator Büchi-B390 (BÜCHI Labortechnik AG, Switzerland). The concentration of sodium alginate was constant (1.5% w/v), whereas initial calcium chloride concentrations varied from 0.5 to 2.0 mol dm-3. Microspheres (ALG/Ca or ALG/(Ca+*Tv*))were formed in the cross-linking solution under mechanical stirring, then washed several times with sterilized water, filtered through Büchner funnel and stored at 4 oC until further studies.

MC was prepared by coating of MS with chitosan layer, that is by alginate and chitosan complexation. 50 grams of washed microspheres (ALG/Ca or ALG/(Ca+*Tv*))were dispersed in 100 ml of chitosan solution (0.5% CS in 1.0% CH3COOH) under constant stirring. Microcapsules without (CS/(ALG/Ca) or with *Tv* (CS/(ALG/(Ca+*Tv*))were filtered, washed with deionized water and phosphate saline buffer, and stored at 4oC until further studies.

*2.3. Methods*

*2.3.1. Fourier transform infrared spectroscopy analysis*

The Fourier transform infrared spectroscopy (FTIR) spectra were recorded with the FTIR Instrument - Cary 660 FTIR (MIR system) spectrometer (Agilent Technologies, USA). Samples were mixed with potassium bromide to get pellets. Spectral scanning was done in the range of 500-4000 cm-1.

*2.3.2. Microscopic observations*

MS and MC size, morphology and topography were analyzed by several microscopic techniques: (i) optical microscopy (OM) (Leica MZ16a stereo-microscope, Leica Microsystems Ltd., Switzerland), (ii) scanning electron microscopy (SEM) (FE-SEM, model JSM-7000F, Jeol Ltd., Japan)), (iii) confocal laser scanning microscopy (CLSM) (TCP SP2, Leica Lasertechnik, Germany) and (iv) atomic force microscopy (AFM) (Bruker Billerica, USA).

The average diameter of wet and dry microparticles was determined by optical microscopy using Olympus Soft Imaging Solutions GmbH, version E\_LCmicro\_09Okt2009. Twenty microparticles were randomly selected from batches produced in triplicate, to determine the size distribution.

Confocal laser scanning microscopy was operated in transmitted and fluorescent mode at an acceleration voltage of 80 kV. All sample preparations were performed at room temperature. MC was stained with eosin (0.01%) and placed on a microscope slide and sealed.

Microparticles for SEM analysis were put on the high-conductive graphite tape. FE-SEM was linked to an EDS/INCA 350 (energy dispersive X-ray analyzer) manufactured by Oxford Instruments Ltd. (UK). The ImageJ software was used for the determination the size of pores on a microparticle surface.

The samples for AFM imaging were prepared by deposition of a microparticle suspension on the mica substrate. The microparticles are flushed three times with 50 μl of MiliQ water in order to remove all residual impurities. The microparticle surface, cross section and grain size distribution within each sample were analyzed using MultiMode Scanning Probe Microscope with Nanoscope IIIa controller (Bruker, Billerica, USA) with SJV-JV-130V ("J" scanner with vertical engagement); Vertical engagement (JV) 125 μm scanner (Bruker Instruments, Inc.); Tapping mode silicon tips (R-TESPA, Bruker, Nom. Freq. 300 kHz, Nom. spring constant of 40 N/m). In this manner, three-dimensional information about the surface topology was obtained and the roughness was quantified. All AFM imaging was performed at three different regions of each microparticle to ensure consistency of obtained results.

*2.3.3. Swelling degree and fraction of released bioactive agents*

Detailed procedures for the determination of swelling degree and fraction of released *Tv* and calcium ions from microparticles were previously described (Vinceković et al., 2016).

The swelling degree (Sw) was calculated using the equation:

(1),

where *w*t is the weight of the swollen MS or MC, and *w*0 is their initial weight. All measurements were replicated three times and results are presented as the mean values.

*In vitro* release studies from microparticles were carried out at room temperature. Results are presented as the fraction of released *Tv* or calcium ionsusing the equation:

(2),

where f represents the fraction of *Tv* (f*Tv*)or calcium ions(fCa) released, Rt is the amount of *Tv* or calcium ionsreleased at time t, and Rtot is the total amount of *Tv* or calcium ionsloaded in microparticles.

The concentration of *Tv* (expressed as the number of spores (NS) per 1 g of dry microparticles) was determined at λ =550 nm and the concentration of calcium cations at λ = 650 nm using UV-VIS spectrophotometer (Shimadzu, UV-1700). The release studies were carried out at the room temperature in triplicate for each formulation and standard deviations were calculated.

*2.3.4. Statistical analysis*

The results were statistically analyzed with Microsoft Excel 2016 and XLSTAT statistical software add-in. The data are shown as mean values ± standard deviation.

**3. Results and discussion**

*3.1. Identification of intermolecular interactions in microparticles*

FTIR spectra of MS prepared at various calcium chloride concentrations revealed functional groups of all components interact with each other. Complex intermolecular interactions include mainly electrostatic interactions and hydrogen bonds. It was shown that the increase in calcium cation concentrations increases the crosslinking degree of calcium alginate microspheres, whereas the loading with *Tv* diminishes crosslinking degree due to the electrostatic repulsions between negatively charged *Tv* and free parts of alginate chains as well as mechanical interactions (Jurić et al., 2019).

FTIR spectra of MC prepared at various calcium chloride concentrations are given in Figure 1. The strong and broad absorption band between 3500 and 3000 cm−1 recorded in all spectra belongs to the hydroxyl (–OH) groups stretching vibrations and to the amine (–NH2) groups superimposed on the hydroxyl groups due to the presence of chitosan coating layer (spectrum of ALG and CS are displayed in Figure 1 for comparison). Besides bending vibration for –NH2 groups superimposed on the hydroxyl, several characteristic peaks (at 1650 cm−1 for –CO stretching, at 1555 cm−1 for –NH2 bending vibrations, at 1370 cm-1 for asymmetric –COC and –CN stretching and 1150 cm-1 for –CH3 symmetrical deformation) disappeared or becomes weaker due to interaction between or superposition of the functional groups of chitosan and alginate (Sankalia, Mashru, Sankalia & Sutariya, 2007). The absence of chitosan band at 1582 cm-1 (–NH bending vibration), reduced intensity and shifting of asymmetric (1595 cm-1) and symmetric (1405 cm-1) alginate carboxylate bands indicates electrostatic interactions between two oppositely charged polyelectrolytes (the –NH3+ of the chitosan with the –COO- of the alginate) (Vinceković et al., 2016).

An increase in calcium cations concentration causes the most significant changes in the alginate functional groups region (hydroxyl and carboxylate) as was previously shown for microspheres (Jurić et al., 2019). As the concentration of calcium ions increases the intensity of absorption band around 3400 cm-1 decreases from 0.5 to 1.5 mol dm-3 and then increases at 2 mol dm-3. Difference in the intensity of absorption band between MS Jurić et al., 2019) and MC indicates changes in intensity of hydrogen bonds between oxygen atoms of G-residues and the calcium in the egg-box structure. Decrease in the intensity of absorption band around 3400 cm-1 indicates the diaxially linked guluronic acid residues form smaller cavities which accommodate a lesser amount of water (Shi, He, Teh, Morsi, & Goh, 2011; Roy, Bajapi, & Bajapi (2009).

In both, MS and MC, the sodium alginate asymmetric and symmetric carboxylate peaks became broader exhibiting gradual intensity increasing and shifting of carboxylate ions stretching vibrations (asymmetric to a lower and symmetric carboxylate vibrational peak to a higher wavenumbers) with increasing calcium cation concentrations.

Changes in some *Tv* characteristics bands are an indication of at least interactions with hydroxyl, amine, carboxylate and carbonyl groups between *Tv*,alginate and calcium ions as was similarly observed for interactions in microcapsules prepared with copper ions as gelling cation (Vinceković et al., 2016). Changes in FTIR spectra of MS and MC clearly show the presence of chitosan layer differently influences on their structures with increasing calcium cation conentration.



**Fig. 1.** FTIR spectra of CS/(ALG/(Ca+*Tv*)) microcapsules prepared at different initial calcium chloride concentration, *ci*(CaCl2)/mol dm-3 = 0.5 (red line), 1.0 (blue line), 1.5 (green line) and 2.0 (black line). Spectra of *Tv* (olive line), chitosan (gray line) and alginate (yellow line) are given for comparison.

*3.2. The viability of encapsulated Trichoderma viride spores in microparticles*

Our recent investigation has shown the interactions between calcium cations and negatively charged *Tv* stimulated germination during storage inside electrostatically cross-linked networks of alginate microspheres (Jurić et al., 2019). Fig. 2. presents change in the number of spores loaded in microparticles with time (1, 10 and 20 days). There was no significant change in NS between freshly loaded and 1 day stored in microparticles. During storage for 10 days, the NS increased by approximately 21% and 15% in MS (Jurić et al., 2019) and 10% and 7% in MC in microparticles prepared at 1.0 and 1.5 mol dm-3 of calcium chloride, respectively. During storage within 20 days, the NS somewhat decreased although remained still higher than in freshly prepared microparticles. The best biomass yield was observed in microparticles prepared at 1 mol dm-3 of initial calcium chloride concentration. Experiments confirmed alginate microparticles prepared with calcium chloride provide an environment supportive of *Trichoderma viride*sporulation, although the presence of chitosan on the surface somewhat reduced the yield of spores.

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**Figure 2.** Change in the number of *Tv* (NS/g) in ALG/(Ca+*Tv*) microspheres (empty signs) (Jurić et al., 2019), and CS/(ALG/(Ca+*Tv*)) microcapsules (filled signs) prepared at initial calcium chloride concentration, *ci*(CaCl2) = 1 (○,●), 1.5 (□,■), with time (t). The error bars indicate the standard deviation of the means.

*3.3. Size and morphology of microparticles*

Size, size distribution and shape optical microscopy observations revealed allprepared microcapsules were almost spherical, but after drying to constant mass (approximately four weeks on air at the room temperature) their sphericity was lost. The increase in calcium concentration from 0.5 to 2.0 mol dm-3 resulted in a decrease of MC size (approximately 16%)as was also observed for MS (Jurić et al., 2019). This is in accordance with literature data showing the formation of smaller microparticles at higher CaCl2 concentrationsdue to a lower percentage of water retained and formation of more rigid gels (Daemi & Barikani, 2012).

Wet MC prepared with *Tv* (CS/(ALG/Ca+*Tv*)) were slightly larger than those prepared without *Tv* (CS/(ALG/Ca)) implying change in the network structure due to the electrostatic repulsions between negatively charged *Tv* (Vinceković et al., 2017) and free parts of alginate chains (the zeta potential of the calcium alginate matrix is about -10 V) (Rokstad, Lacik, de Vos, & Strand, 2014)as well as mechanical interactions. The addition of *Tv* resulted in the size increase around 6, 10, 13 and 16% in investigated calcium concentration range. As was observed for MS (Jurić et al., 2019), dried MC was also approximately two times smaller than the wet as a consequence of water and humidity loss associated with biopolymer strain-relaxation processes.

Microphotograph of a wet MC taken under CLSM in fluorescence mode (Fig. 3a) clearly shows the existence of thin chitosan layer on the surface. Chitosan layer became visible by staining with eosin, which due to its anionic character specifically binds to amino groups of chitosan.In contrast to MC prepared without *Tv* showing smooth surface (Fig. 3b), the surface of those prepared with *Tv* (Fig. 3c)exhibit numerous oval dimples (diameter around 3.6 µm) and a germ tube crossed the surface of microcapsule within a few days. Sections close to the surface revealed the presence of hyphae (Fig. 3d) and inside the matrix formation of mycelium (Fig. 3e). Enlarged microphotograph shows details of hyphae growing inside a microcapsule (Fig. 3f). All results clearly show encapsulated spores are able to germinate, that is calcium alginate microparticles with a cross-linked network containing a large fraction of water provide an environment supportive for *Trichoderma viride*germination. Presence of *Tv* and growth of mycelium inside the microparticle obviously change the gel network structure. The results are in accordance with investigations of De Jaeger, de la Providencia, Rouhier, & Declerck (2011) who confirmed the ability of fungi to regrowth outside the calcium alginate coating and to colonize a susceptible plant.

|  |  |  |
| --- | --- | --- |
|  |  |  |
| 1. 100 µm | 1. 25 µm | 1. 25 µm |
|  |  |  |
| 1. 75 µm | 1. 25 µm | 1. 10 µm |

**Fig. 3.** CLSM microphotographs of (a) part of CS/(ALG/(Ca+*Tv*)) microcapsule with chitosan layer (red) taken in fluorescence mode; (b) surface of CS/(ALG/Ca) and (c) CS/(ALG/(Ca+*Tv*)) with protruding germ tube, (d) sections of CS/(ALG/(Ca+*Tv*)) close to the surface, (e) branched hyphae growing inside CS/(ALG/(Ca*+Tv*)), (f) enlarged picture of germ tubes inside CS/(ALG/(Ca*+Tv*)) taken in transmitted mode. Microcapsules were prepared at initial calcium chloride concentration, *ci*(CaCl2) = 0.5 mol dm-3. Bars are indicated.

The surface morphology of dried MS and MC observed by SEM are shown in Figures 4a-f. The surface of MS prepared without *Tv* are highly porous with a pore size of approximately 0.169 μm (Fig. 4a), whereas those prepared with *Tv* (Fig. 3b) exhibits numerous spherical blebs close to the size of individual spores (~3.6 μm) (Jurić et al., 2019). Striped and fibrous surfaces representing the chitosan layer can be seen on MC surface (Fig. 4c). In comparison with MS, the MC surface porosity is reduced. Loading of MC with *Tv* resulted in a sleeker surface with the appearance of numerous dimples (Fig. 4d). Enlarged images (Fig. 4e,f) shows the structure of assembled holes from which the germ tubes penetrate out of the MC.

|  |  |
| --- | --- |
|  |  |
| (a) | (b) |
|  |  |
| (c) | (d) |
|  |  |
| (e) | (f) |

**Fig. 3.** SEM surface microphotographs of microspheres (a) ALG/Ca and (b) ALG/(Ca+*Tv*), and microcapsules (c) CS/(ALG/Ca) and (d,e,f) CS/(ALG/(Ca+*Tv*)). Microparticles were prepared at initial calcium chloride concentration, *ci*(CaCl2) = 0.5 mol dm-3. Bars are indicated.

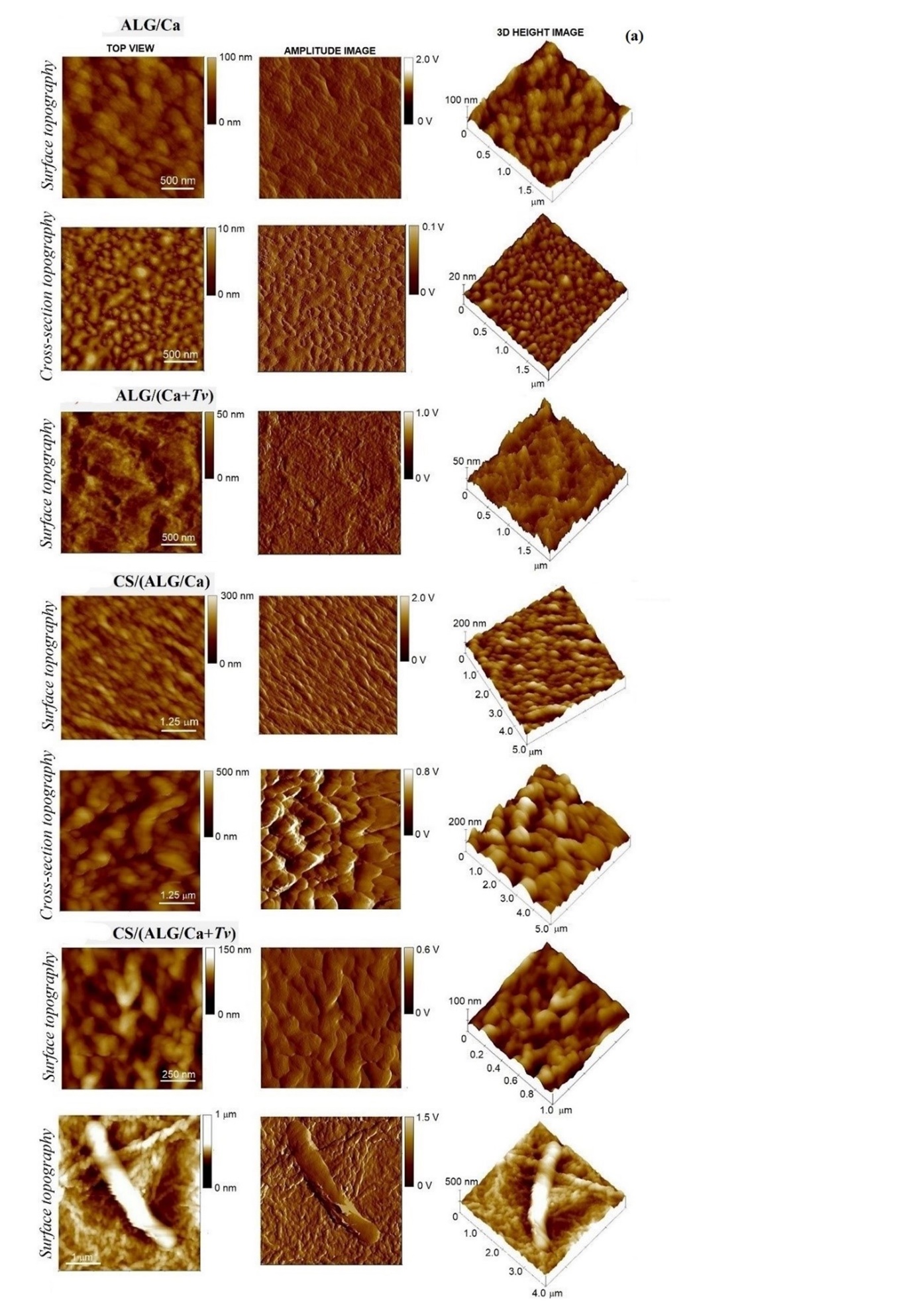
A representative AFM morphological images of microparticles are presented in Figure 5a. AFM analysis of a single MS (ALG/Ca) and MC (CS/ALG/Ca) prepared without *Tv* are given for comparison. The surface area of the ALG/Ca is granular with clearly visible individual grains of height around 30 nm. On the same surface, the topographic image of a larger resolution indicates the subordinate structure of an individual grain. The topographic image of the cross-section clearly shows the finer grain morphological characteristics. The grains are smaller with a height of 2-5 nm and have no subordinate structure.

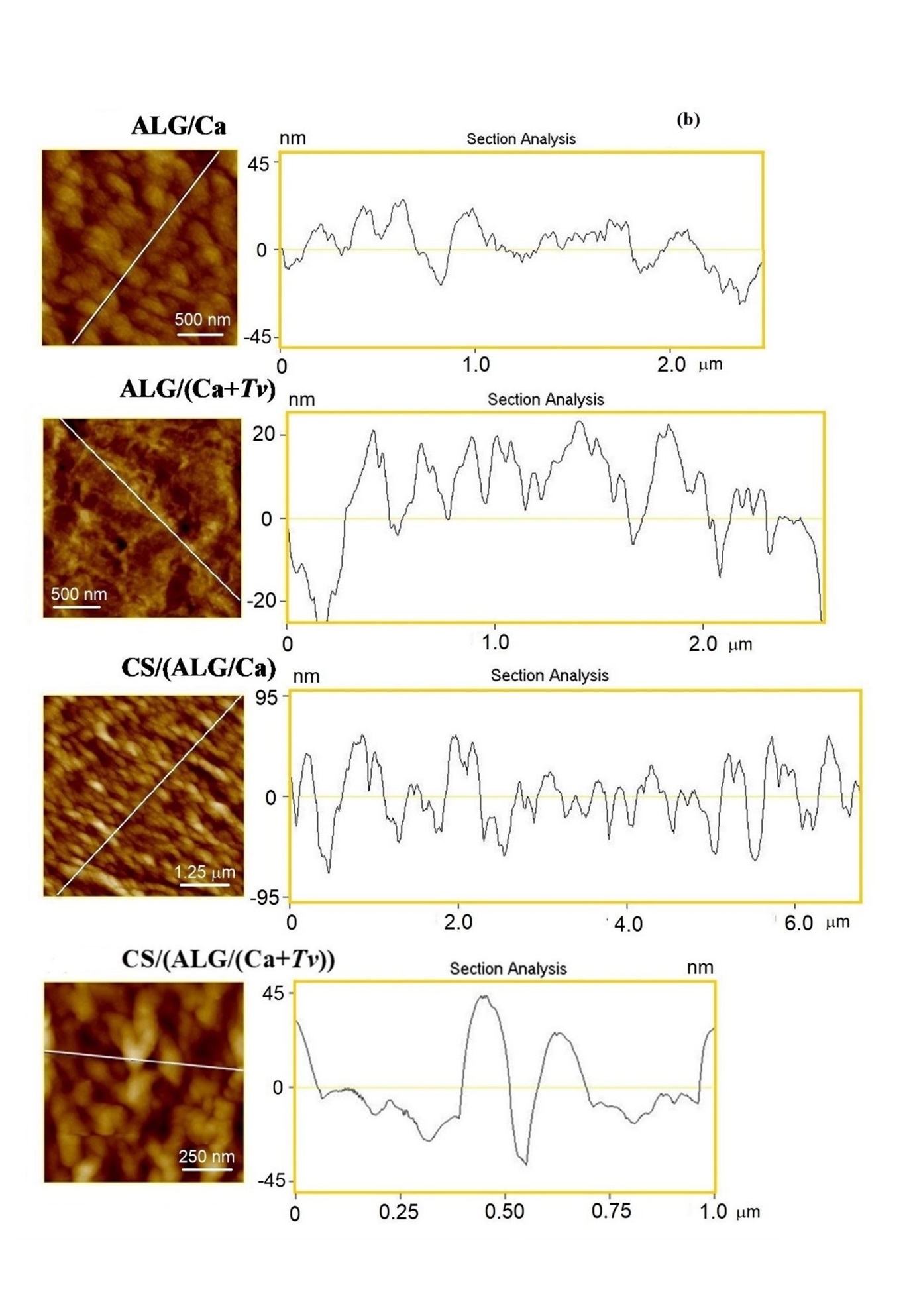
Due to the presence of *Tv*, the surface of ALG/(Ca+*Tv*) is smoother (roughness decreased from *R*a=12±1 nm (ALG/Ca) to *R*a=7.6±0.8 nm (ALG/(Ca+*Tv*)) and loses the grain structure forming a networked fine structure whose height of the cross-linked fibers reaches up to 40 nm. On the same surface, the topographic image of a larger resolution indicates a subordinate structure within the network with a grain structure. The height of each individual grain reaches up to 10 nm.

Images of a single CS/(ALG/Ca) clearly shows a granular structure. The height of grains ranged from 30 nm to 100 nm showing a subordinate structure of an individual grain. The topographical image of the inner cross-section clearly shows different morphological characteristics of the grains. They have no subordinate structure and are bigger than on the surface itself (average grain surface reached 122±143 nm) and protrude above surface resulting in increased roughness of *R*a=26±3 nm.

Loading with *Tv* reduced the surface roughness from *R*a=26±3 nm (CS/(ALG/Ca)) to *R*a=16±3 nm (CS/(ALG/(Ca+*Tv*)). The enlarged surface of the CS/(ALG/(Ca+*Tv*)) revealed the presence of *Trichoderma viride* hyphae. Its presence does not affect the morphological characteristics of grains whose height is up to 5 nm. Both microcapsules, with and without *Tv* have bigger grains (average grain surface reached 427±129 nm) (Table 1), but grains are immersed in the matrix revealing in the decreased roughness value *R*a=16±3 nm.

Surface profiles taken along the white lines in images are plotted in Figure 5b. All microparticles exhibit granular surface with substructures consisting of abundant smaller particles. The average size and mean diameter of grains on the surface and cross-section of microparticles are given in Table 1. A grainy surface texture with significant variation in microroughness suggests a porous morphology. It is visible that the chitosan layer on microcapsules increases surfaces roughness, but loading with *Tv* reduces the surface roughness of both, microspheres and microcapsules (Table 1).





**Fig. 5**. (a) 2D- (TOP VIEW) and 3D-height and amplitude images of ALG/Ca and ALG/(Ca+*Tv*), CS/(ALG/Ca) and CS/(ALG/(Ca+*Tv*)). (b) Surface profiles (right) corresponding to the surface characteristics along the white line (left) of microparticles. Microparticles were prepared at initial calcium chloride concentration, *ci*(CaCl2) = 0.5 mol dm-3.

**Table 1**

Evaluated data of grain size (average and mean diameter) of microparticles: (a) surface and (b) cross-section prepared at initial calcium chloride concentration, *ci*(CaCl2) = 0.5 mol dm-3.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Microparticle | Number of grains | Average/nm2 | Mean diameter/nm | Roughness/nm |
| (a) *Surface* |  |  |  |  |
| ALG/Ca | 80 | 33±20 | 24±37 | 12±1 |
| ALG/(Ca+*Tv*) | 159 | 79±110 | 21±59 | 7.6±0.8 |
| CS/(ALG/Ca) | 89 | 122±143 | 87±129 | 26±3 |
| CS/(ALG/(Ca+*Tv*)) | 18 | 427±129 | 46±21 | 16±3 |
| (b) *Cross-section* |  |  |  |  |
| ALG/Ca | 384 | 26±16 | 24±47 | 2.21±0.03 |
| CS/(ALG/Ca) | 79 | 15±8 | 64±27 | 20±4 |

*3.5. Swelling*

When microparticles were dispersed in water they swell. The increase in calcium concentration decreased the degree of swelling of all microparticles (Fig. 6) The concentration of calcium ions determines the kinetics of alginate gelation and the characteristics of the gel formed (Selimoglu & Elib, 2010). During gelation, the carboxylate groups of guluronic acids react with calcium ions to form a crosslinked network of alginate chains. A higher CaCl2 concentration resulted in a higher crosslinking degree of network and a denser structure, which caused a less swelling when microparticles were dispersed in the solution. In comparison with MS, MC exhibited higher swelling degree due to the hydrophilic nature of chitosan-alginate complex on the surface.



**Fig. 5.** Swelling degree (Sw) variation of microspheres ALG/(Ca+*Tv*) (○) and microcapsules CS/(ALG/(Ca+*Tv*)) (●) with initial calcium chloride concentrations, *ci*(CaCl2). The error bars indicate the standard deviation of the means.

*3.6. Mechanisms and kinetics of Trichoderma viride and calcium release from microparticles*

Design of controlled delivery systems involves the optimization of many parameters (preparation technique, microparticle chemical composition, geometry and size, the conditions during release etc.) among which are the most important type and concentrations of both, biopolymer and gelling cation (Irmanida, Devi, Kusdiantoro, Wahono, & Esthi, 2012). Having in mind all of the factors involved in the release and the combinatorial effect of different microparticle composition and the concentration of cross-linking cation(Rodrigues & Lagoa, 2005) we have studied bioactive agents release from microparticles differing in surface morphology and structure.

It is known the most important rate-controlling release mechanisms from hydrophilic polymer microparticles are diffusion, swelling and erosion,that is the mechanisms and kinetics of release are determined by the rate of diffusion, swelling or erosion (Siepmann & Siepmann, 2012). According to Korsmeyer-Peppas model(Korsmeyer, Gurny, Doelker, Buri, & Peppas, 1983) different controlling mechanisms may be distinguished by a simple empirical equation:

(3),

where *k* is a kinetic constant characteristic for particular system considering structural and geometrical aspects, *n* is the release exponent representing the release mechanism, and *t* is the release time. Values of *n* < 0.43 indicates the release is controlled by classical Fickian diffusion, *n* > 0.85 is controlled by Type II transport, involving polymer swelling and relaxation of the polymeric matrix. Values of *n* between 0.43 and 0.85 show the anomalous transport kinetics determined by a combination of the two diffusion mechanisms and Type II transport.

Figure 7a presents comparison of *Tv* release profiles for MS(Jurić et al., 2019) and MC with increasing calcium cations concentration. All release profiles are characterized by rapid initial release (burst effect) and followed by slower release. They were analyzed by modified Eq. 3,

(4),

where *a* is the y-axis intercept characterizing the burst effect (Lindner & Lippold, 1995; Kim & Fassihi, 1997).

The fraction ofreleased *Tv* from microspheres above 1 indicates a higher concentration of *Trichoderma viride* biomassthen loaded in the microsphere. The increasing amount of *Trichoderma viride* biomass in the surrounding medium is closely related to two sources, one is the release from microspheresand the other is germination. Microscopic observations confirmed such a conclusion revealing the growth of mycelium inside the microparticle matrix and germ tubes protruding toward water phase. Coating of alginate microspheres with chitosan reduces the rate and amount of released *Tv* due to the additional barrier that slows transport from the MC to the surrounding solution (Wang & Zhao, 2013; Wang et al., 2014; Lopez, Maudhuit, Pascual-Villalobos, & Poncelet, 2012; Lucinda-Silva, Salgado, & Evangelista, 2010).



**Fig. 7**.(a)Fraction of released (a) *Tv* (f*Tv*) and (b) calcium cations (fCa) with time (t) from ALG/(Ca+*Tv*) microspheres (empty signs) and CS/(ALG/(Ca+*Tv*)) microcapsules (full signs). Microparticles were prepared at initial calcium chloride concentration, *ci*(CaCl2)/mol dm-3 = 0.5 (○,●), 1.0 (□,■), 1.5 (Δ,▲) and 2.0 ( **▽**, ▼ ). The error bars indicate the standard deviation of the means.

The values of the intercept *a*, release constants *k* and exponents *n* are listed in Table 2. A decrease in the release rate with increasing calcium chloride concentration is in accordance with the effect of gelling cation concentration on the strength of the alginate network structure. Values of *n* between 0.43 and 0.85 would point to the controlling release mechanisms from MS as anomalous transport kinetics (a combination of diffusion mechanisms and Type II transport). Obtained *n* valuesclose to or higher than 0.85 for MC indicate the release is controlled by Type II transport, that is by the polymer swelling and relaxation (transition of glassy structure to a rubbery state).

**Table 2**

Variation of the y-axis intercept (*a*), the release constant (*k*/h), exponent (*n*), and correlation coefficient (R2) of *Tv* released from ALG/(Ca+*Tv*) and CS/(ALG/Ca+*Tv*)) at the initial calcium chloride concentration, *ci*(CaCl2)/mol dm-3.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *ci*(CaCl2) | *a* | *k* | *n* | R2 |
| ALG/(Ca+*Tv*)\* |  |  |  |  |
| 0.5 | 0.124 | 0.155 | 0.46 | 0.98 |
| 1.0 | 0.227 | 0.059 | 0.52 | 0.99 |
| 1.5 | 0.507 | 0.025 | 0.64 | 0.99 |
| 2.0 | 0.550 | 0.025 | 0.70 | 0.99 |
| CS/(ALG/(Ca+*Tv*)) |  |  |  |  |
| 0.5 | 0.541 | 0.0035 | 0.80 | 0.98 |
| 1.0 | 0.597 | 0.0038 | 0.81 | 0.99 |
| 1.5 | 0.612 | 0.0032 | 0.85 | 0.99 |
| 2.0 | 0.620 | 0.0015 | 0.96 | 0.99 |

\* Jurić et al., 2019

When calcium alginate microparticles are dispersed in water various physicochemical processes such as wetting, swelling (penetration of solution into the matrix) and polymer stress relaxation (transition of glassy structure to rubbery state), diffusion through the matrix, disintegration, dissolution or erosion of the structure, or their combination, may be included in the release of an active agent from a hydrophilic microparticle. Undoubtedly, all these processes somewhat collapse the structure of a microparticle and it is also expected the releasing of calcium ions. Calcium is an essential plant macronutrient and promotes *Trichoderma viride* germination. Its release along with released *Trichoderma viride* will have a beneficial impact on the plants, that is, they will act simultaneously.

Therelease of calcium cations from microparticles with an increasing concentration of calcium cations is presented in Figure 7b. All release profiles are characterized by rapid initial release followed by slower release obeying a power law equation. To identify the kinetics and type of mechanism involved in the release Eq. 3 was applied. Values of *n* < 0.43 indicate that the release process of calcium cations is controlled by diffusion through microparticles. In the measured time intervals both, MS and MC released a very small fraction of calcium. As is expected the amount and the rate of released calcium ions from MC is smaller due to the presence of polyelectrolyte complex layer on the MC surface.

**Table 3**

Variation of the release constant (*k*/h), exponent (*n*) and correlation coefficient (R2) of calcium released from ALG/(Ca+*Tv*) microspheres and CS/(ALG/(Ca+*Tv*)) microcapsules prepared at initial calcium chloride concentration, *c*i(CaCl2)/mol dm-3.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| *c*i(CaCl2) | *k* | *n* | R2 | *k* | *n* | R2 |
|  | ALG/(Ca+*Tv*)\* |  |  | CS/(ALG/(Ca+*Tv*)) |  |  |
| 0.5 | 6.5x10-4 | 0.07 | 0.99 | 3.1x10-4 | 0.07 | 0.98 |
| 1.0 | 6.1x10-4 | 0.04 | 0.98 | 2.9x10-4 | 0.04 | 0.98 |
| 1.5 | 5.9x10-4 | 0.03 | 0.99 | 2.5x10-4 | 0.05 | 0.99 |
| 2.0 | 5.2x10-4 | 0.02 | 0.99 | 2.4x10-4 | 0.06 | 0.98 |

\* Jurić et al., 2019.

The release from alginate microparticles prepared at the lowest calcium cation concentration was the fastest and decreased with increasing calcium chloride concentration. This may be ascribed to the influence of cation concentration on the gelation rate which controls microparticle homogeneity and percentage of retained water (Rodrigues & Lagoa, 2006). At lower cation concentration gelation is slower producing more uniform structures with a high percentage of water. The diffusion of calcium ions through brittle gels is faster than in stiffer gels formed at higher gelling cations concentration. The obtained differences in the kinetics can be attributed to the effects of calcium cations concentration on the microparticle structure as well as to the ability of calcium binding to *Tv* (Jurić et al., 2019). The presence of chitosan layer on the microcapsule surface further slows down the calcium cation release.

**4. Conclusions**

The presented results contributed to better understanding of the relationship between alginate microparticle structure and bioactive agents release properties. Calcium cation concentration, presence of *Tv* and chitosan layer affect microparticle surface morphology and structure*.* Besides theeffect of increasing calcium cation concentration, the presence of *Tv* and growth of mycelium inside the microparticles change the gel network structure. Surface of microparticles is granular with substructures consisting of abundant smaller particles. Microcapsules exhibited bigger grains and higher surface roughness than microspheres. Loading with *T. viride* reduced the surface roughness forming sleeker surface with holes from which the germ tubes penetrated out of microparticles. The mechanism of *Tv* release from microspheres is a combination of diffusion mechanism and the Type II transport (polymer swelling and relaxation). *Tv* release from microcapsules is controlled mainly by Type II transport indicating bigger surface grains on microcapsule surface slows down processes of the polymer swelling and relaxation. Differences in microparticle surface properties and structure do not affect the controlling mechanism of calcium cations release (detected as diffusion through microparticles) but influence the rate of calcium release. Both, the rates of *Tv* and calcium released from microcapsules are smaller due to the presence of chitosan layer on the surface.

Better understanding of relationship between structural properties of a microparticle and kinetics and mechanisms controlling the release of active agents enhances the ability to control their release behavior and may aid in developing new microparticles with tailored properties. Recently, we have applied variously designed microparticles simultaneously loaded with chemical and biological agents on several plants under greenhouse conditions and in an open field. After the treatments, grapevine vine leaves reached a significant increase in bioactive potential compared to the control (Vinceković et al., 2019). The results obtained on the other plants (lettuce, tomato, strawberries, tobacco) are very promising and their publication is under preparation.

**Author Contributions**

The corresponding author designed the research work and wrote the manuscript. Other authors carried out work under the guidance of the corresponding author.

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