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Control of colloidal CaCO_3 suspension by using biodegradable polymers during fabrication

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ABSTRACT

Fabrication of homogenous CaCO_3 particles is a significant step in assembling poly-electrolyte capsules. It is crucial to control the dimensions, the shape and the charge of the calcium carbonate particles in order to have homogeneously separated and charged templates as final result. For this reason, previously, they have been deeply investigated.

Recently, crystallization of CaCO_3 was done by adding poly (sodium 4-styrenesulfonate) (PSS) as negatively charged polymer and poly (allylamine hydrochloride) (PAH) as positively charged polymer and the results were surprising. The homogenous particles were separated and they carried the same charge of the used polymer.

The aim of this work was to investigate the synthesis process of CaCO_3 particles in different experimental conditions: calcium carbonate was produced in presence and in absence of water and with addition of appropriate polymers. In particular, chitosan (CHI) and poly acrylic acid (PAA) were chosen as biodegradable polymers whereas PSS and PAH were chosen as non-biodegradable polymers. Shape and diameter of particles were investigated by using transmission and scanning electron microscopy, elemental composition was inferred by energy dispersive X-ray analyses whereas their charges were explored by using zeta potential.

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1. Introduction

Synthesis of CaCO_3 has been an attractive issue since many years (Ogino et al., 1987; Spanos and Koutsoukos, 1998; Koga et al., 1998; Tracy et al., 1998; Horn and Rieger, 2001; Kitamura 2001; Kitamura et al., 2002) because it provides an

excellent template for encapsulation of cargo molecules either by using co-precipitation in its porous (Fig. 1, Scheme 1, pre loading method) (Volodkin et al., 2004) or by loading cargo molecule after core removal (Fig. 1, Scheme 2, post loading method) (Sukhorukov et al., 2001). Furthermore, it is not toxic and can be easily and gently removed by complexation with

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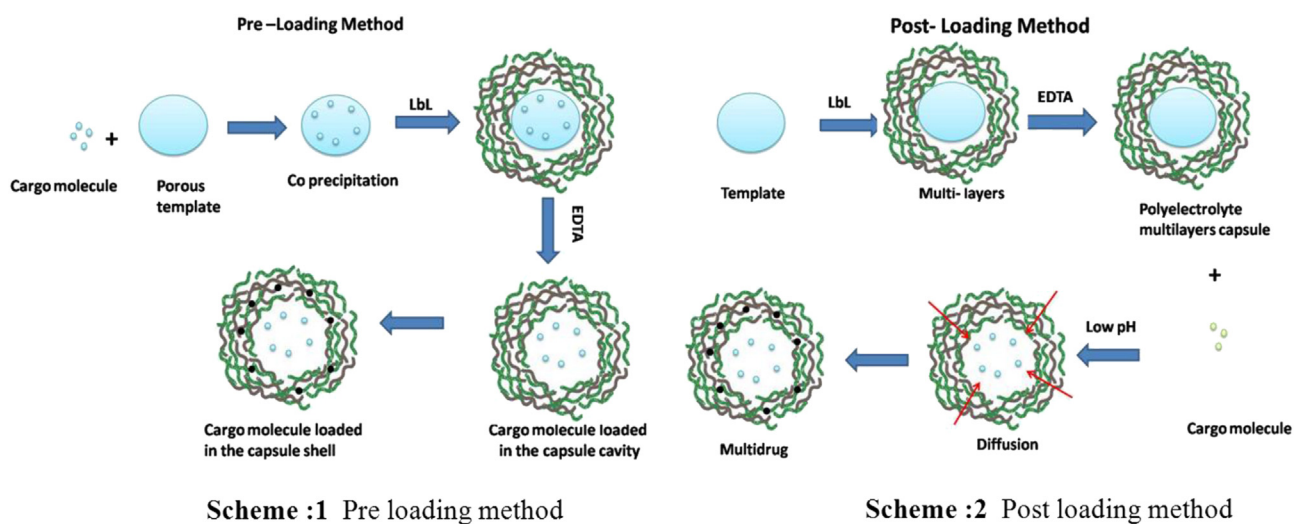


Fig. 1 – Schemes for drug encapsulation.

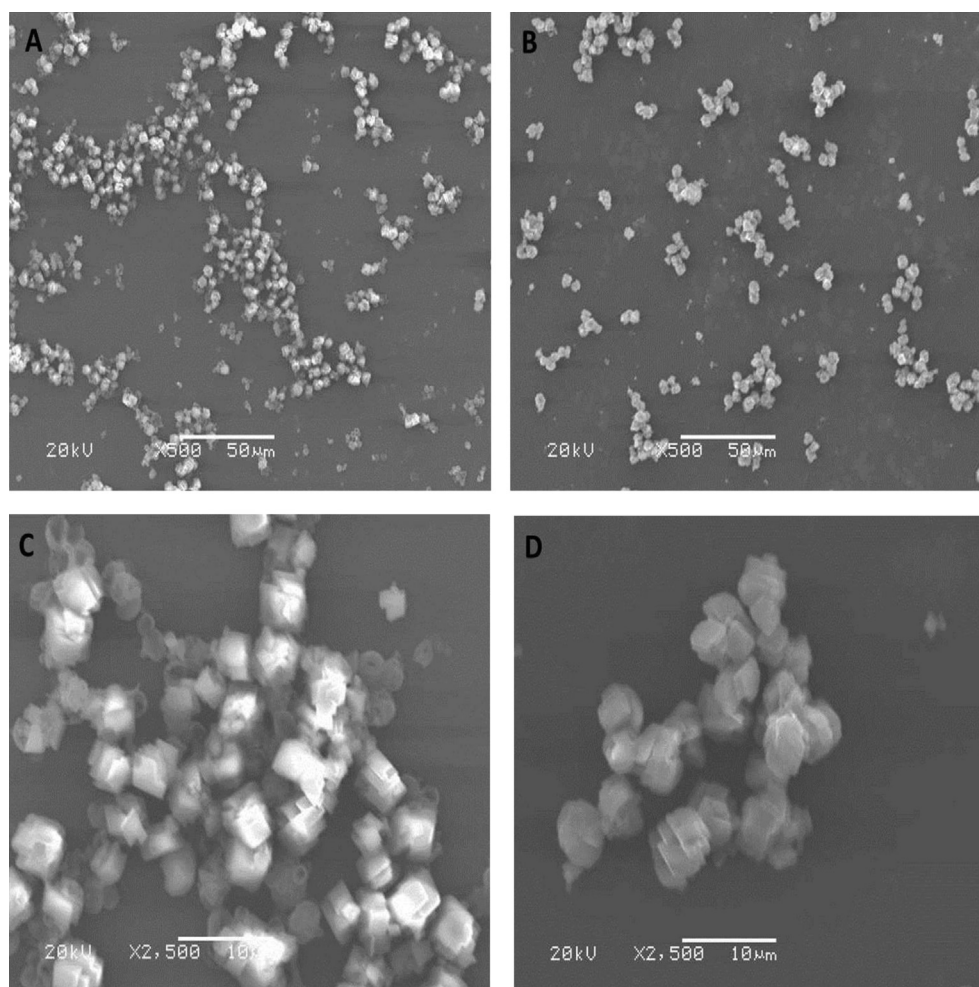


Fig. 2 – SEM images of CaCO_3 particles fabricated in absence (A and C) and in presence of distilled water (B and D). The high magnification (2.500X) shows aggregated particles in both samples (C and D).

ethylendiaminetetraacetic acid (EDTA) (De Koker et al., 2011). The natural porosity of CaCO_3 microparticles has also the ability to build matrix during layer-by-layer (LbL) assembly. Polymers, used in polyelectrolyte multilayer assembly, will be adsorbed in these porous-forming interior polyelectrolyte network complexes having similar chemistry as the shell after dissolving the core (Sukhorukov et al., 2004a, b; Volodkin et al., 2007). This network matrix-type is important for the final capsule building against environmental conditions, such as temperature and humidity. Moreover, this network type might give capsule mechanical improvement to store it for long time. Additionally, the network matrix might be important for capsule structure in blood stream. The obtained capsule quality is closely related to the quality of prepared template.

Previously, crystallization of CaCO_3 was done by adding PSS as negatively charged polymer and PAH as positively charged polymer (Jiafu et al., 2013) giving to capsules unique properties in drug selection according to interior core charge of the used polymer. Diameter of particles produced by PSS was arranged from 2 to 8 μm (Tong et al., 2005) that was obtained by mixing PSS with CaCl_2 and then by adding Na_2CO_3 to

the solution. The produced particles were homogenously separated and negatively charged. The same properties were found in particles produced in presence of PAH (Jiafu et al., 2013).

At the moment, reducing the diameter to submicron scale with the same polymers is difficult, so the possibility to control the fabrication of CaCO_3 by using biodegradable polymers was studied in this work. For this reason, four polymers have been chosen: PSS and PAA as negatively charged polymers and PAH and CHI as positively charged polymers. Each polymer was prepared separately and incubated with Na_2CO_3 for 5–10 min before mixing with CaCl_2 .

2. Material and methods

2.1. Chemicals

The suppliers of the chemicals were as follows: poly (sodium 4-styrene-sulfonate) (PSS), poly (allylamine hydrochloride) (PAH), protamine salt, grade III (PRM), poly acrylic acid (PAA) and ethylenediaminetetraacetic acid disodium salt dihydrate

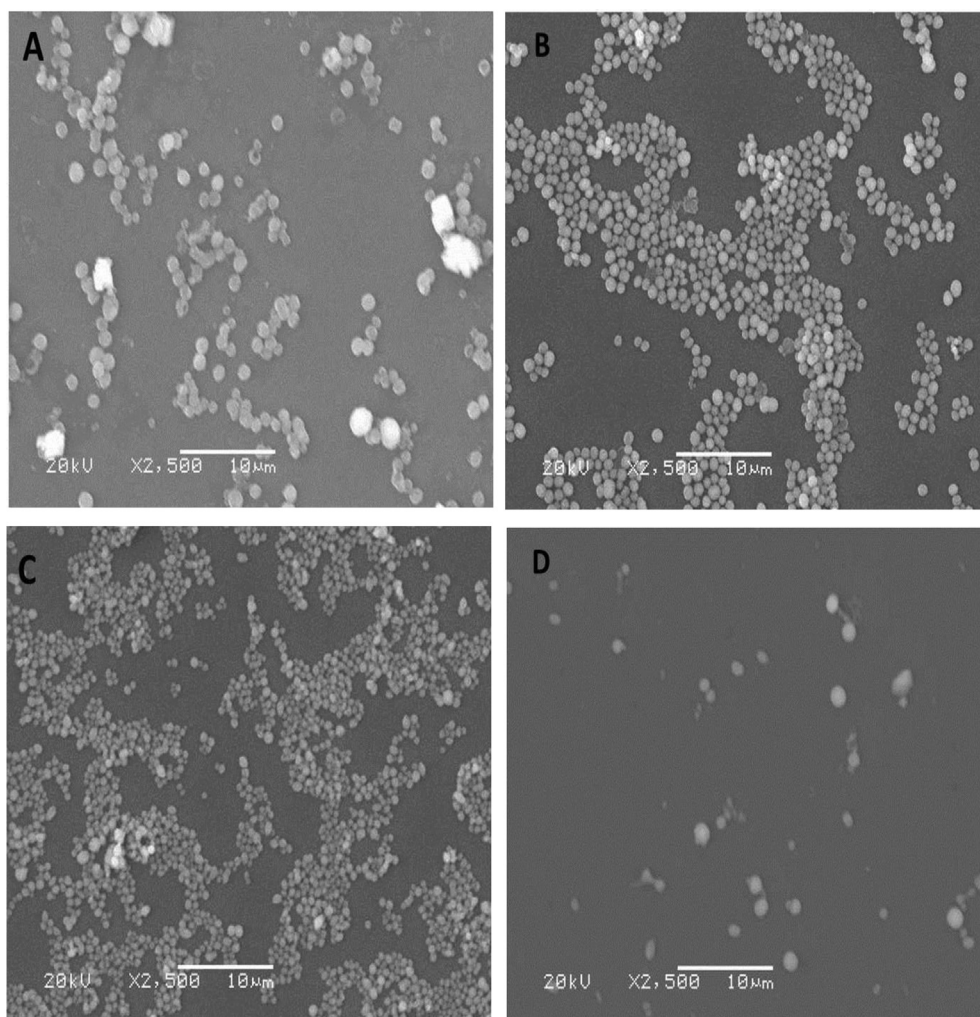


Fig. 3 – SEM images of CaCO_3 particles prepared in presence of polymer: (A) CHI; (B) PSS, (C) PAA and (D) PAH. Vaterite shape is the most common shape in all samples.

99% were purchased from Sigma, USA; dextran sulfate sodium salt (DEX) from Sigma, *Leuconostoc* spp., Germany; poly acrylic acid (PAA), chitosan (CH) and calcium chloride dehydrate 99,99% ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) from Aldrich, USA; sodium carbonate (Na_2CO_3) from Merck, Germany.

2.2. Polymers preparation condition

The concentration of polymers was decided after several previous experiments in our laboratory. So, the final concentration was set to 10 mg/1 ml d.w. for each polymer. Chitosan was dissolved in the same concentration by using 1% acetic acid.

2.3. Fabrication of CaCO_3 particles

Calcium carbonate particles (CaCO_3) were fabricated with same molar used by Volodkin et al. (2004). Briefly, 750 μl of polymer was taken in a glass bottle and well mixed with 615 μl of 0.33 M Na_2CO_3 and then 615 μl of 0.33 M $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ was added rapidly under magnetic stirrer for 30 s. Afterwards the solution was left for 3 min without stirring for sedimentation. CaCO_3 particles were collected by centrifug at 5000 rpm for 1 min and washed

three times by using Milli-Q Water. The procedure results in highly homogenous, spherical CaCO_3 microparticles with an average diameter ranging from 100 nm to 1.5 μm . Calcium carbonate particles were fabricated in three different conditions: in distilled water, without distilled water and in polymer. The same volume was used in polymer and in distilled water.

2.4. Fabrication of PRM/DEX multilayers on polymers doped CaCO_3 template

Protamine and dextran were assembled onto the core alternatively in 0.5 M NaCl solution for 20 min (3 min sonication plus 17 min shaking) followed by three-stages washing in Milli-Q water. The concentration of each polymer was 5 mg/ml. The excess of polyelectrolyte was removed by centrifugation at 2000 rpm for 3 min. After assembling the layers, capsules were obtained by dissolving the core in 0.2 M EDTA (pH 5.5) solution shaking for 30 min, followed by three-stages washing by using 0.2 M EDTA (pH 7.2). Capsules were centrifuged under low speed (800 rpm for 15 min) to prevent aggregation and they were washed three times in Milli-Q water.

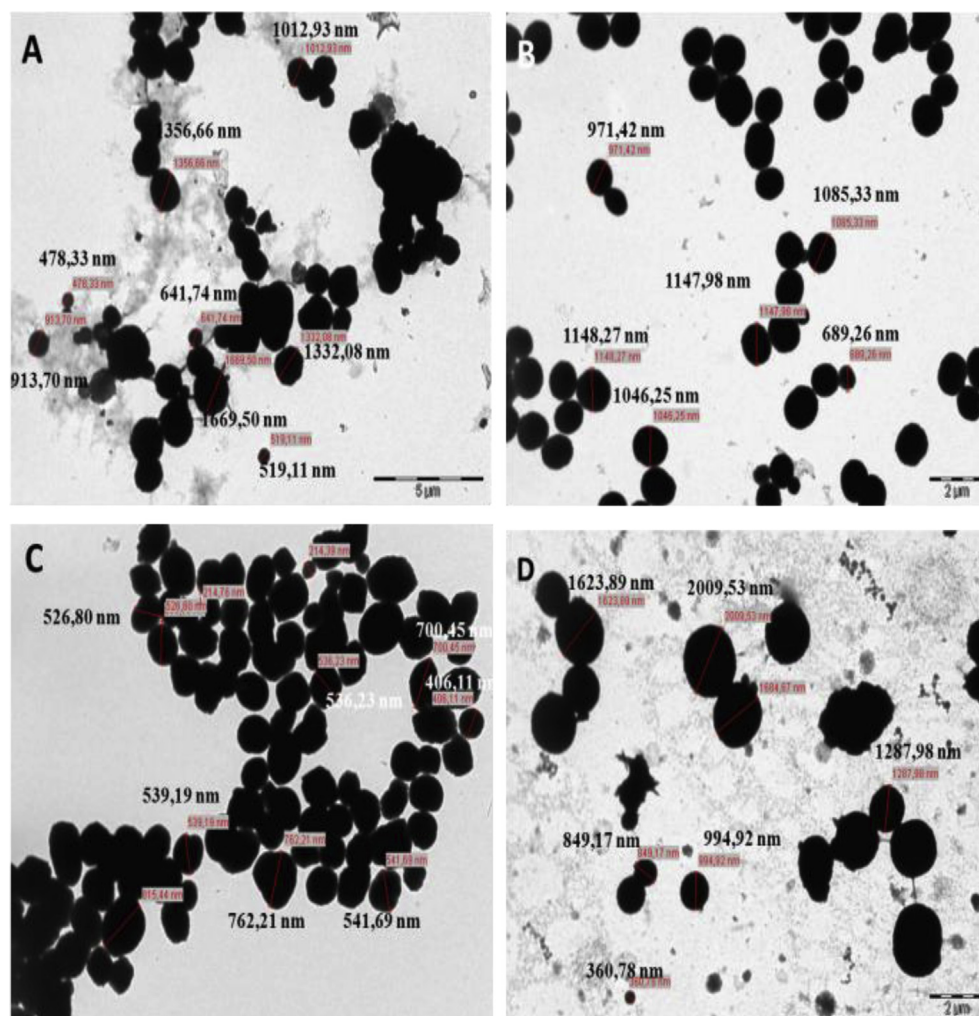


Fig. 4 – TEM images of CaCO_3 particles fabricated in presence of polymers: (A) CHI, (B) PSS, (C) PAA and (D) PAH.

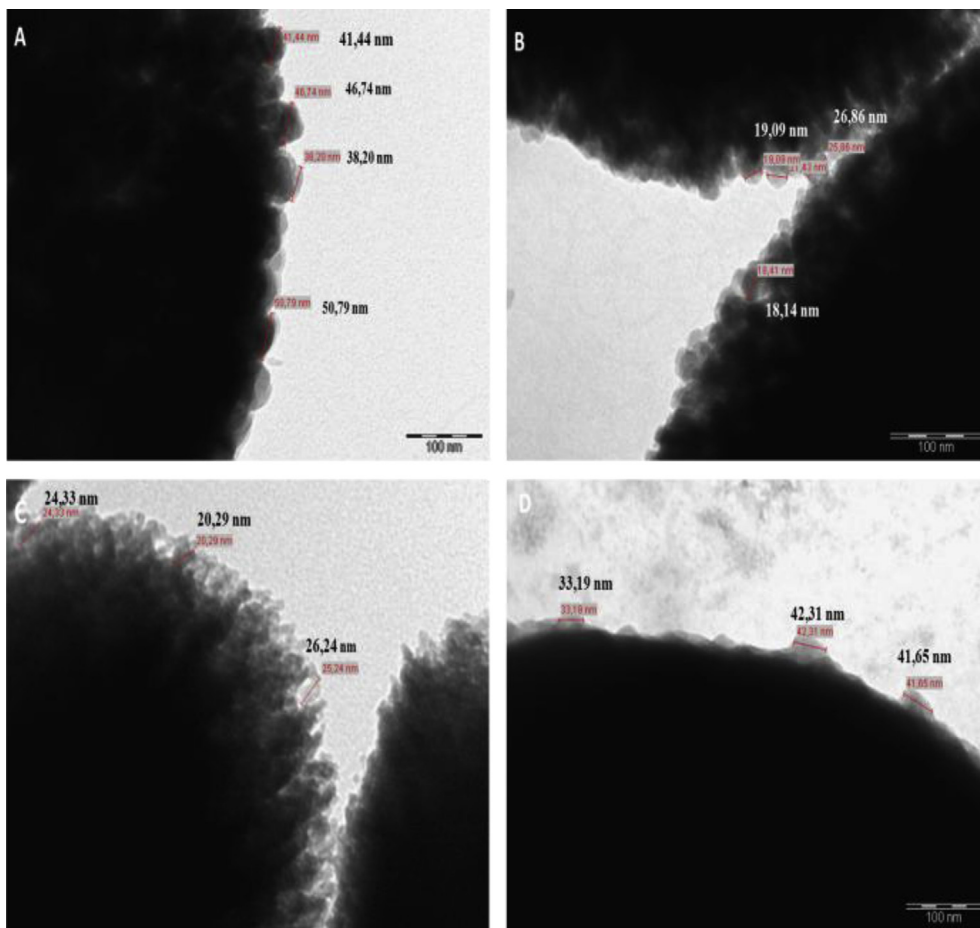


Fig. 5 – High magnification TEM images of CaCO_3 particles in presence of polymers: (A) CHI, (B) PSS, (C) PAA and (D) PAH.

2.5. Scanning electron microscopy (SEM)

For SEM analysis, few drops of samples of the particle suspension were dropped onto a SiO_2 wafer and then dried overnight. Samples were sputtered with a 5 nm gold layer and measurements were conducted under 5 kV-accelerating potential electron beam by using SEM (JEOL JSM-6480LV). Images were processed by using the software SEM/JSM 5000.

2.6. Elemental analysis distribution

The same sample preparation of SEM was used for elemental analysis. Elemental distribution was inferred from Energy-Dispersive X-ray (EDX) measurements (IXRF analyser 5000).

2.7. Transmission electron microscopy (TEM)

For TEM analysis, 10 μL of each sample suspension were deposited on the copper grid and air-dried before measurements. Copper grids, sputtered with carbon films, were used to support the sample. Samples were analysed by a JEOL JEM 1011 operating at 100 kV, coupled with a GATAN camera ORIUS SC600 with a resolution of 7 Megapixel. The GATAN camera is controlled by Digital Micrograph.

2.8. Electrophoretic mobility

The electrophoretic mobility of particles and LbL-coated microparticles was measured by using a Malvern Nano ZS90 (Malvern Instruments, UK). The mean of five consecutive runs were taken for particles and after adsorption of each layer.

3. Results

3.1. Characterization of calcium carbonate

CaCO_3 particles prepared without and with water were analysed by SEM (Fig. 2). In both cases images shows multifaceted particles which tend to aggregate while samples prepared in

Table 1 – Summary of crystals/particles charge and size.

Polymer	Crystal size	Particle size	Particle charge (mV)
CHI	38–50 nm	400 nm–1.6 μm	-1.8 ± 0.6
PSS	18–25 nm	600 nm–1.1 μm	-16.4 ± 0.4
PAA	19–25 nm	400 nm–600 nm	-27.2 ± 0.8
PAH	28–35 nm	82 nm–3 μm	18.1 ± 0.8

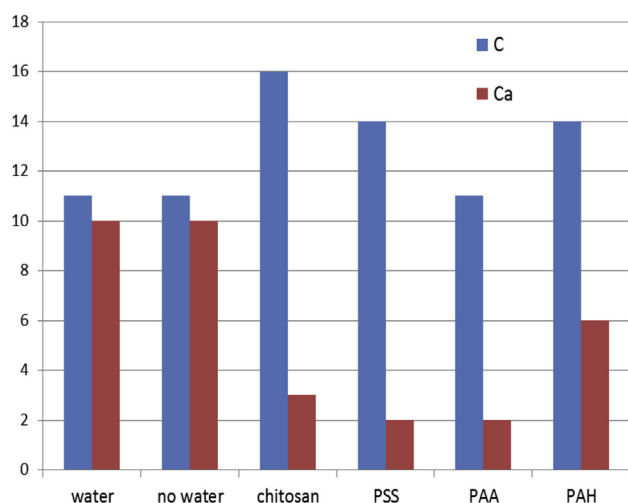


Fig. 6 – Elemental distribution of calcium and carbon atoms in different conditions.

presence of polymers exhibits spherical, separated and homogenous population as shown in (Fig. 3). Additionally, vaterite is the main shape in all the samples.

3.2. Size of calcium carbonate

Diameters of calcium carbonate particles prepared in presence and absence of distilled water were detected through

TEM measurements and are in a range between 4 and 5 μm while those obtained in presence of polymers have diameters ranging from 82 nm to 3 μm (Fig. 4). A remarkable difference in diameter is observed for particles produced in various polymers. PAA has strongly reduced particle diameters to nano-scale (400 nm–600 nm). While diameter of particles prepared by using PSS were between 600 nm and 1.1 μm . Particles prepared by using PAH showed different diameters ranging from 82 nm to 3 μm . While particles prepared by CHI were ranged from 400 nm to 1.6 μm . Concerning crystals size of particles (Fig. 5) prepared with CHI and PAH, they were in the ranges 38–50 nm and 28–35 nm, respectively, being larger than those prepared with PSS and PAA which are in the ranges 18–25 nm and 19–25 nm, respectively. Crystals and particles sizes are summarized in Table 1.

3.3. Elemental distribution

Elemental analysis for distribution of calcium and carbon atoms shows differences in particles prepared in presence of polymers compared to particles prepared in presence and absence of water. In case of particles prepared in presence and absence of water, atomic concentration of calcium and carbon atoms are about at the same level (ca 10% and 11%, respectively, see Fig. 6). On the contrary, calcium concentration is lower than carbon concentration when particles are produced in presence of polymers. In particular, calcium and carbon atomic concentrations were about 3% and 16% in CHI, 2% and

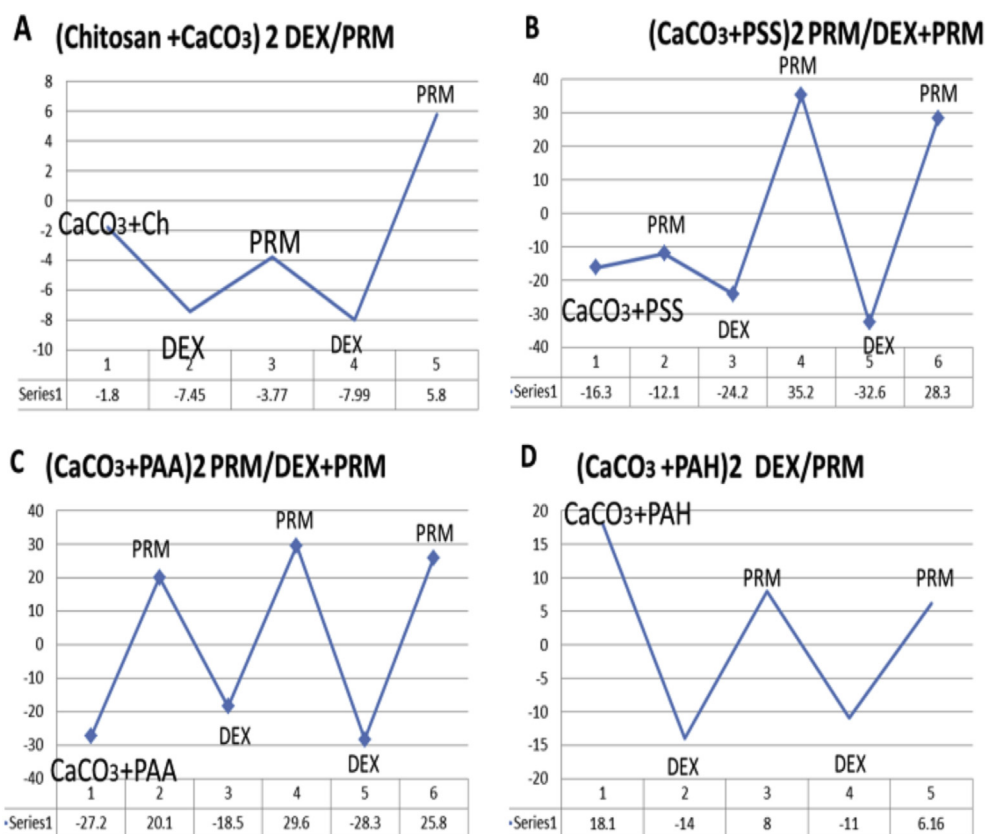


Fig. 7 – Zeta potential measurements of coated CaCO₃ particles after each polymer used (A) CHI, (B) PSS, C) PAA and (D) PAH.

Table 2 – Physical properties of each polymer used for particle fabrication.

Polymer	Description	Ions	Type of crystal	Active group
CHI Positive	Biodegradable polysaccharide	Semi-movable	Vaterite and few calcite	Amino group
PSS Negative	Not-biodegradable	Movable	Vaterite and few calcite	Carboxylic group
PAA Negative	Biodegradable	Complete stable	Vaterite and few calcite	Carboxylic group
PAH Positive	Not-biodegradable	Stable	Elongated vaterite and flower shape	Amino group

14% in PSS, 2% and 11% in PAA and 6% and 14% in PAH, respectively.

3.4. Physical properties of used polymers

Zeta potential measurements indicate that ions are more stable in particles prepared by using PAA (see Fig. 7C), indeed strongly adsorption is detected in the first layer and after each layer of PRM/DEX, compared to particles prepared by using PSS (cfr Fig. 7B) because its ions have affected adsorption of first and second layers. CHI dissolved in acetic acid showed slightly negative charged particles (Fig. 7A). Finally, PAH particles showed good adsorption of negative layers during coating (Fig. 7D). Physical properties of polymers are summarized in Table 2.

3.5. Electrophoretic mobility

Zeta potential shows alternation of positively and negatively charge values which are related to the used polymer (Table 1). Strong negative charge is observed in particles prepared in PAA (-27.2 ± 0.8 mV) compared to particles in PSS (-16.3 ± 0.4 mV), while positive charge is observed in particles prepared in presence of PAH (18.1 ± 0.8 mV). CHI particles shows slightly negative charges (-1.8 ± 0.6 mV) because CHI was prepared in acetic acid.

4. Discussion

The direct mixing of soluble salts of Ca_2^+ and CO_3^- results in an amorphous CaCO_3 precipitate (Sukhorukov et al., 2004a, b)

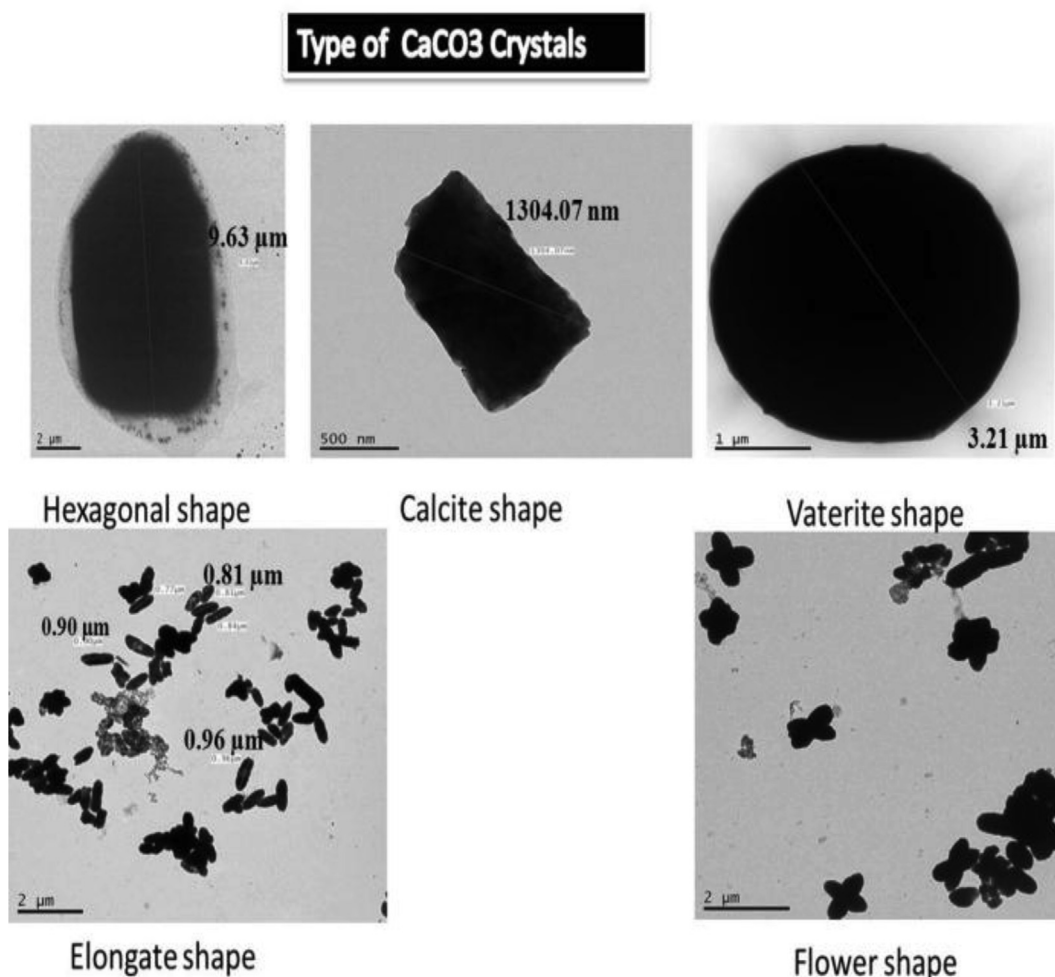


Fig. 8 – TEM images of different type of CaCO_3 crystals after their fabrication.

with several polymorphic forms, ranked in order of decreasing thermodynamic stability: calcite, aragonite and vaterite (Sawada, 1998). Other crystal shapes like elongated and flower-shaped structure (Liang et al., 2010) were also observed (see Fig. 8). These different CaCO_3 particles of polymorphic forms and particle sizes can significantly affect their final application (Babou-Kammoe et al., 2012). For this reason, CaCO_3 crystallization shapes should be reduced to spherical vaterite shape that is stable under LbL assembly, forming finally capsules that have the same shape as shown in Fig. 9. Additionally, vaterite particles have many favourable properties such as biocompatibility, high drug-loading capacity and payload protection (Sukhorukov et al., 2004a, b; Peng et al., 2010; Volodkin et al., 2010). Since cellular uptake behaviour is largely influenced by particle size, the control of diameter and shape of template plays an important role to improve drug delivery carrier.

Single particle of CaCO_3 is formed by tiny smaller crystals. They are attached together by electrostatic force. If there is no electrostatic balance, tiny crystals tend to bind with other crystals in the same solution causing aggregation and growth in diameter. Furthermore, they are not easily separated by

using mechanical separation (e.g., vortex and shaker or even physical separation like ultrasound sonication) (Fig. 10, Scheme A).

The control of parameters affecting the rate of the nucleation process became a way of improving the final template. The quality of the resultant microparticles is found to be strongly dependent on the experimental conditions such as the type of the used salts, their concentration, pH, temperature, rate of mixing solutions and the intensity of agitation of the reaction mixture (Kitamura et al., 2002; Yu et al., 2004; Wang et al., 2006; Babou-Kammoe et al., 2012). Inclusion of different additives, such as divalent cations, organic solvents and macromolecules (synthetic or natural) added to the reaction mixture, are shown to exert a strong effect on the morphology of the formed CaCO_3 microparticles (Kato et al., 1998; Manoli and Dalas, 2000; Colfen and Qi, 2001; Parakhonskiy et al., 2013).

Previously, during CaCO_3 synthesis researchers have used organic materials and its derivatives that could strongly influence the crystallization of CaCO_3 ; stabilized amorphous CaCO_3 could be formed due to functional groups of organic material leading to control nucleation, growth,

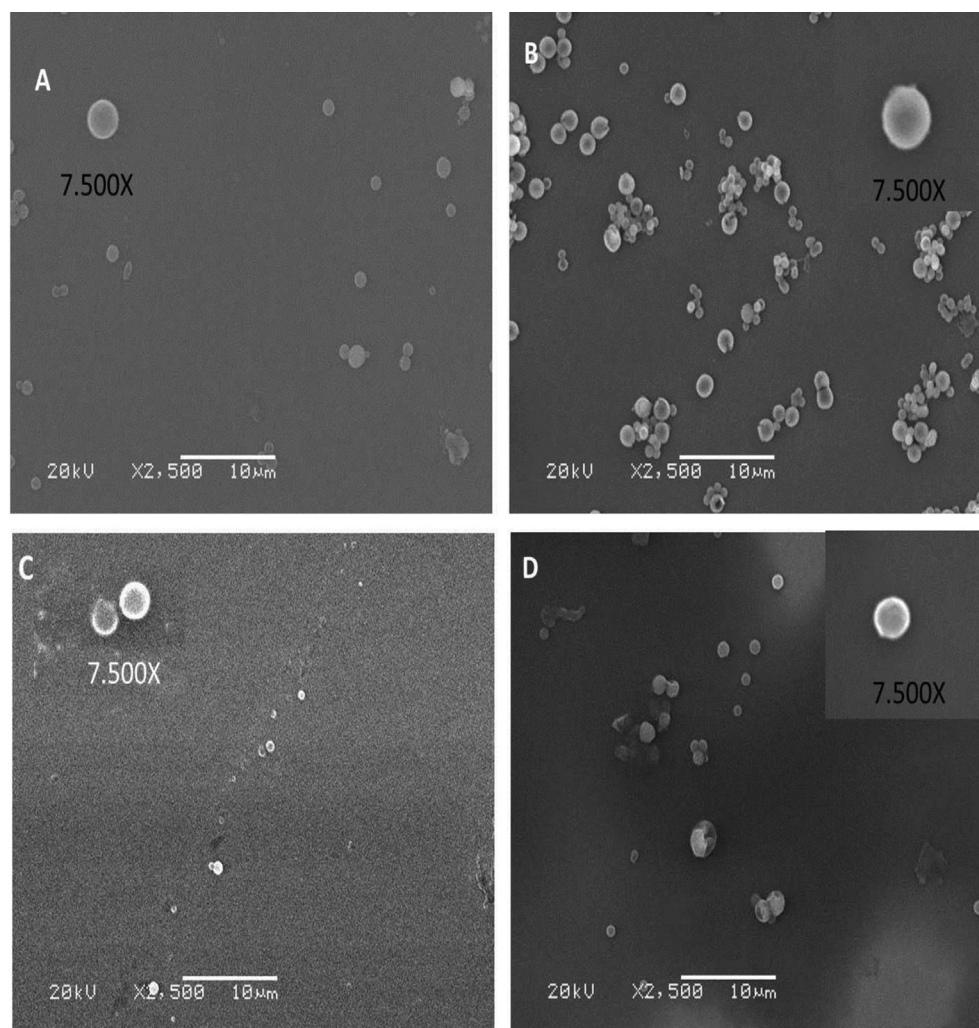


Fig. 9 – SEM images of not aggregated capsules in each used polymer after dissolving the core. (A) CHI, (B) PSS, (C) PAA and (D) PAH-doped CaCO_3 capsules. Insets (7.500X magnification) show single hollow capsule for each sample.

and alignment of the crystals (Didymus et al., 1993; Gower and Tirrell, 1998). Recently, simple polyelectrolyte have been used to control the crystallization of carbonates during synthesis (Jada and Verraes, 2003). Specially, PSS (Tong et al., 2005; Xu, 2013) and PAH (Jiafu et al., 2013) are used in our work. Both polymers are initially incorporated during fabrication of the CaCO_3 crystal matrix and then are entrapped inside the microcapsules after core removal giving to particles with unique properties. They are negatively or positively charged related to the type of used polymer. This method might produce detached particles, improve morphological properties, give rise to a more homogenous population and reduce the hexagonal shape from population, being calcite and vaterite the main shapes in CaCO_3 crystallization. PAA, as negatively charged polymer, gives the same results, leading to separated and charged particles as shown by SEM (see Fig. 3) and TEM (crf Fig. 4). Besides, CHI has slightly negatively charged because it dissolved in acetic acid.

Furthermore, the different polymers determine the characteristics of the multilayer polymer assembly (Fig. 7). The first layer of coating is determined according to type of the

particle charge. In case of negatively charged particles, a cationic polymer is used for coating, whereas in case of positively charged particles, an anionic polymer is used. Additionally, for particles prepared in presence of polymers, CaCO_3 crystals dissolved completely by using EDTA solution because of the formation of lines inside the matrix of crystal acting as barrier between tiny crystals, leading to prevention of crystal agglomeration inside matrix of particle (Fig. 10, Scheme B).

Separated CaCO_3 crystals, after PSS addition, are influenced by functional groups on their long hydrophilic chains, which can complex with Ca^{2+} and disperse them to avoid agglomerating when calcium carbonate particles are growing (Jin et al., 2009). In the same way, it acts as precursor for the subsequent mesoscale self-assembly and finally the micron-sized particles are formed (Tong et al., 2005).

Elemental EDX analysis of calcium and carbon atoms is a good indicator for elemental distribution inside particles after CaCO_3 fabrication as shown in Fig. 6. The level of calcium and carbon atoms is the same in particles prepared in presence and absence of water, compared to those particles prepared by using polymers. The atomic elemental distribution can be

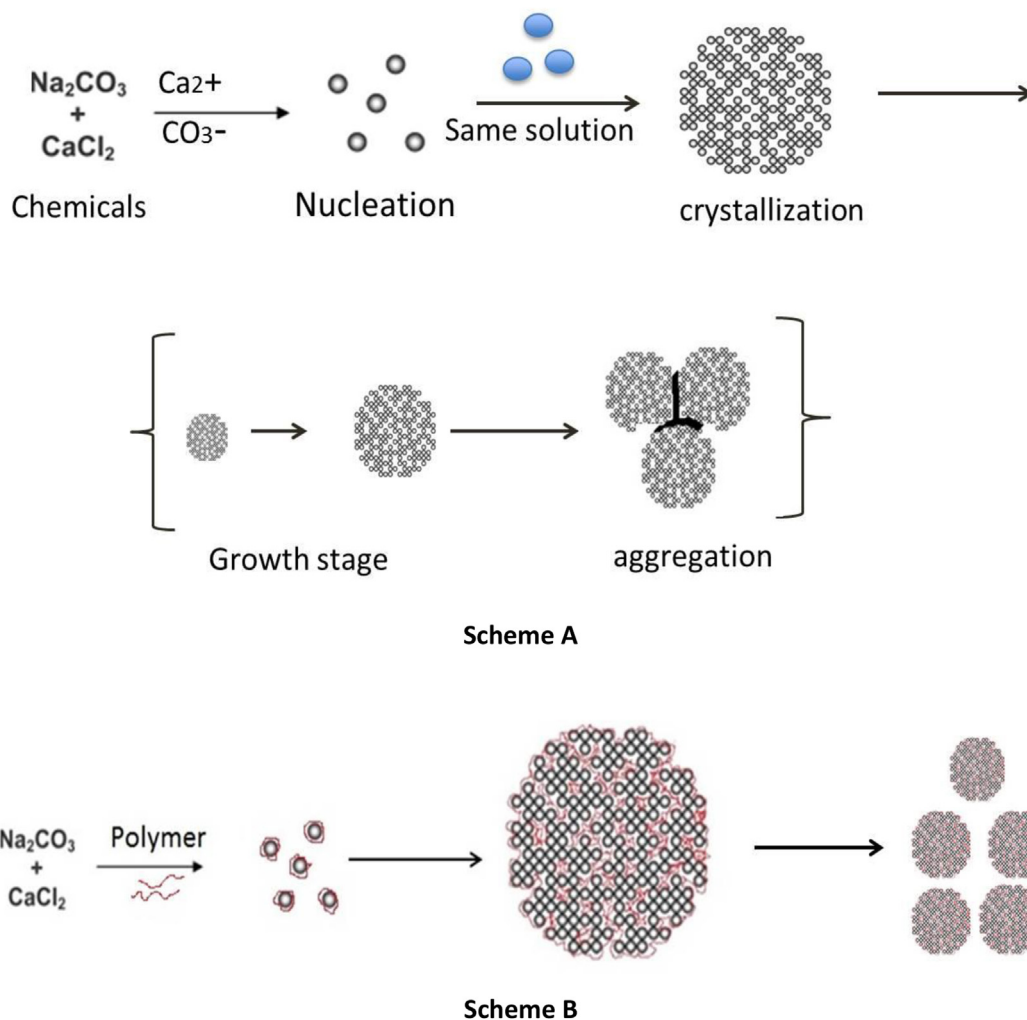


Fig. 10 – Scheme A) Ca^{2+} ions are attached with CO_3^{2-} ions and form CaCO_3 nuclei; Scheme B) polymers are covered by crystal and mixed with nuclei of CaCO_3 .

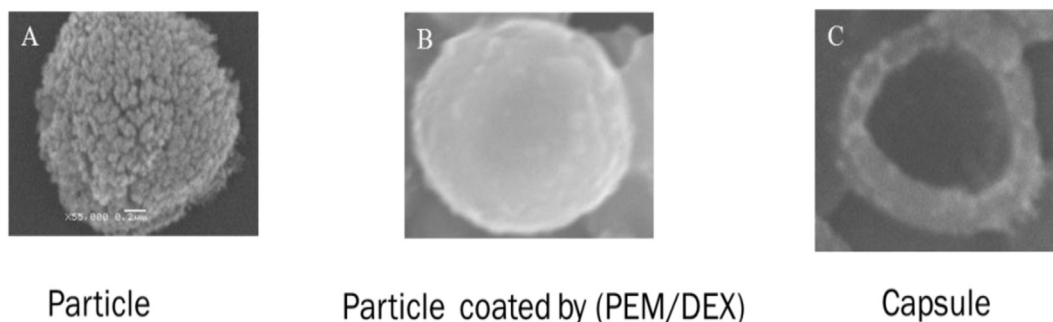


Fig. 11 – SEM images of CaCO_3 particle under the same magnification (55.000x) before and after LbL coating. (A) particle with porous surface. (B) coated particle having smooth surface. (C) hollow capsule with polymer layers visible.

compared to the results obtained by SEM images: when calcium and carbon atoms are distributed at the same level concentration inside the particles, crystal shape is formed (see Fig. 2). In contrast, when concentration of calcium atoms is lower than carbon atoms concentration, vaterite shape is formed (see Fig. 2). These results explain the role of polymers used during CaCO_3 fabrication. Every polymer reduces calcium atoms with respect to carbon atoms. For this reason, spherical vaterite shape is detected in all particles prepared in presence of polymers.

Previous reports (Coradin et al., 2001; Zhang et al., 2003, 2008) have demonstrated that the addition of PAH, a cationic polymer, during the co-precipitation of CaCl_2 and Na_2CO_3 would result in PAH segregated CaCO_3 microspheres with a porous structure. Therefore, PAH as positively charged polymer has the same role of PSS in separation of CaCO_3 particles. As a rule, the spherical CaCO_3 microparticles turn to rhombohedral calcite microcrystal after several weeks of storage in water at room temperature because of re-crystallization (Volodkin et al., 2004) while microparticles prepared with PSS have no shape change during successive six months of storage in water (Wang et al., 2006) because negatively charged PSS is adsorbed on the surface of carbonate nanoparticles constituting the porous CaCO_3 microparticles (as seen in Fig. 11) and prevents the re-crystallizations. High stability of the CaCO_3 microparticles in water is very important for the successive LbL assembly. It is noticed that polymer has strong effect on the size of tiny crystal causing smaller size particle, as shown in Table 1. Particles produced by PAA were smaller (400–600 nm) than particles produced by PSS (600–1.1 μm). This means that PAA has strong effect on electrostatic stability of particles preventing their growth. Differently CHI particles have nearly the same size as PSS particles (400 nm - 1.6 μm). So polymers have influenced the generation of crystals during nucleation formation keeping them under electrostatic balance and this has caused growth stabilization.

5. Conclusion

The quality of templating core depends mainly on parameters affecting nucleation during fabrication. Biodegradable polymers (CHI and PAA) are able to control of CaCO_3 nucleation

during fabrication. Biodegradable polymers give electrostatic balance, to nuclei of crystals, preventing crystal size growth and their aggregation. For each polymer, a variation in diameter of CaCO_3 particles, depending on its ability to control size of crystal, is observed by TEM and SEM. Particles produced have the same charge of used polymer except for CHI. In spite of its positive charge, particles synthesized by using CHI are slightly negative because CHI was prepared in acetic acid. Moreover the first layer of the polyelectrolyte multilayer assembly is determined according to charge type of particles.

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