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CALCIUM CARBONATE MICROPARTICLES: FORMULATION, PREPARATION, CHARACTERIZATION, RELEASE OF BIO-MOLECULES AND APPLICATIONS

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Abstract

Administration of pharmaceuticals involves a variety of different formulations of which colloidal drug carrier systems are currently of interest. This review details the properties of calcium carbonate ($CaCO_3$) microparticles, a promising carrier for bio-molecules as biosensors, drug carriers and for enzyme immobilization. A short introduction of $CaCO_3$ microparticles precedes appropriate methods of formulation and preparation. Emphasis is laid on characterization of $CaCO_3$ microparticles and includes determination of particle size, size distribution and morphology using the following methods: scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and scanning (atomic) force microscopy (SFM or AFM). Dynamic light scattering method investigates the zeta potential and electrophoretic mobility whereas solid state characterization involves methods such as differential scanning calorimetry (DSC), X-ray diffraction (XRD) and Fourier transform infrared microscopy (FT-IR). Encapsulation efficiency of the macromolecules, the *in vitro* drug release studies that have been carried out so far as well as the parameters affecting them, and the general *in vivo* fate of administered $CaCO_3$ are also considered.

Keywords: $CaCO_3$ microparticles; Characterisation, Bio-molecules; Encapsulation efficiency; Enzyme release.

INTRODUCTION

The need to develop suitable drug carrier systems in order to localize, control and improve drug delivery has caught pace in this decade. Several particulate carrier systems have been investigated based on lipids and polymers including natural, semi-synthetic and synthetic systems (Muller *et al.,* 2000). With the numerous disadvantages suffered by some of these carrier systems, scientists are never relenting to discover other practical ways to improve, localize or control the delivery of bio-molecules to their target areas of the body. Composite inorganic materials as potential drug carriers are making waves at the moment (Oh *et al.,* 2005; Ueno *et al.,* 2005). They are commonly prepared by conjugation of inorganic materials (especially carbonates of calcium, barium and cadmium) with polymers (López-Arce *et a.,* 2011; Rauschmann *et al.,* 2005; Shen *et al.,* 2004; Pukánszky, 2008).

Calcium carbonate ($CaCO_3$) is a cheap natural mineral forming an

important part of building materials of hard tissues like bones and teeth (Shen *et al.,* 2004; Pukánszky, 2008; Joshi and Srivastava, 2009; Wikesjo *et al.,* 2003; Koo *et al., 2005*). It has also found use as filler in pigment, rubber, paper and plastic industries (Hund *et al.,* 2008; Volodkin *et al.,* 2004). $CaCO_3$ exists in several solid states, three hydrated forms (calcite, aragonite and vaterite) of which calcite is the only thermodynamically stable modification (Brecevic and Kralj, 2007; Loste *et al.,* 2003; Menahem and Mastai, 2008; Wei *et al.,* 2003; Xie *et al.,* 2005). Porous microparticles of $CaCO_3$ have also been widely used for biomedical and pharmaceutical applications owing to its biocompatibility and biodegradability. The ability to dissolve the $CaCO_3$ core in mild conditions of acidic pH and in the presence of complexing agents (thereby generating microcapsules), makes it a very interesting approach for drug delivery (Ueno *et al.,* 2005; Wei *et al.,* 2005).

Polyelectrolyte microcapsules have been introduced as a novel type of nanoengineered multifunctional material (Watanabe and Akashi, 2009; López-Periago *et al.,* 2010; Gao *et al.,* 2005; Radtchenko *et al.,* 2000; Radziuk *et al.,* 2007). Permeability as a function of the wall materials of the microcapsules have received increasing attention due to an increased interest in their applications such as catalysis, biotechnology, food, environment and medicine, especially in drug delivery (Angelatos *et al.,* 2005; Yang *et al.,* 2005; Peyratout and Dähne, 2004; Shchukin *et al.,* 2006; Stein *et al.,* 2006; Gordon *et al.,* 2004; Shchukin *et al.,* 2003). Currently, microcapsule systems have the highest application in the pharmaceutical industry since they satisfy the many requirements to deliver drugs rightly in terms of time, place and concentration (Reibetanz *et al.,* 2006).

The encapsulation of bio-molecules on porous $CaCO_3$ microparicles has been possible by nanoenginered layer-by-layer (LBL) self assembly technique which builds up multiple nano-sized layers on the $CaCO_3$ template using oppositely charged polyelectrolytes. The electrostatic LBL technique which initially was seen as a surface coating, modification and engineering tool all of a sudden extended to utilize colloidal micro- and nanopaticles as the template involving a wide range of substances to construct multilayers for effective encapsulation (Petrov *et al.,* 2005). The greatest advantage of the electrostatic LBL protocol is the stricking simplicity with which the shell thickness can be tuned to nanometric precision by controlling the number of adsorbed molecular layers. Shell thickness and properties of polyelectrolytes allow the permeability of the capsule shell to be manipulated. This allows the encapsulation of macromolecules, proteins, drugs and other bioactive materials into such microcapsules as is widely applied in pharmaceutics and biotechnology in terms of drug delivery, controlled release and as microcontainers for catalysis (Petrov *et al.,* 2005). As a matter of fact, polyelectrolyte capsules (LBL protocol) has necessitated the microencapsulation of bio-substances with various physicochemical properties and biological functionalities (proteins, enzymes, DNA and drugs), and subsequent release from them have been intensively studied in recent years (Petrov *et al.,* 2005). Enzymes can be involved in the multilayer shells of the capsules via alternative adsorption (Volodkin *et al.,* 2004), and such system has been employed as a biosensor (Schüler *et al.,* 2000) which can provide an inert environment for maintenance of bio-functionality and activity of enzymes (Shan *et al.,* 2007). High loading efficiency and stability

are important advantages which promote their usage in biosensor development with capability of detection of analytes in a selective and specific manner. Furthermore, they can serve as instant analysis of biochemicals under real time conditions of near invasive techniques due to their biocompatibility and biodegradability, in the form of a low cost rapid and simple to operate analytical tool. Recently, nanosized $CaCO_3$ has shown promising approach for enzyme immobilization (Schüler *et al.,* 2000; Shan *et al.,* 2007; Trau *et al.,* 2006). Glucose oxidase immobilized on $CaCO_3$ has shown satisfactory analytical performance and high thermal stability (Schüler *et al.,* 2000) where as enzymes encapsulated porous $CaCO_3$ nanoparticles have been proven to intensify enzyme performance (Shan *et al.,* 2007).

The main objective of this review is to describe $CaCO_3$ microparticles, production methods, characterization methods, ability of $CaCO_3$ to be used as a bioactive/drug carrier system and applications of $CaCO_3$ microparticles.

$CaCO_3$ microparticle preparation techniques

Materials

Commonly used materials for $CaCO_3$ microparticles are:

- Inorganic salts including calcium chloride ($CaCl_2.2H_2O$), sodium carbonate (Na_2CO_3).
- Polyelectrolytes: many different polyelectrolytes can be used as presented in Table 1. Sodium poly(styrene sulfonate) [PSS, MW ~ 70 kDa] anionic; Poly(allylamine hydrochloride) [PAH, MW ~ 70 kDa] cationic; Fluorescein isothiocyanate (FITC); Rhodamine B isothiocynate, mixture of isomers (Rh.), cationic, etc.
- Proteins: many different proteins can be used according to Table 1; including dextran, bovine serum albumin (BSA), α-chymotrypsin (cht), lysozyme (Lys), bovine milk, α-lactalbumine, type III (Lact.), chicken's egg lysozyme, horseradish peroxidase, type 1 (Per.), etc.
- Water, prepared in a three stage Millipore Milli-Q Plus 185 purification system with a resistivity higher than 18.2 MΩ.cm or double quartz distilled water.

Generally, the core (template) is a dissolvable colloidal particle, a drug particle, a dye particle or even a biological cell (e.g. droplets, dye and drug particles, erythrocytes and other biological cells, inorganic cores, carbonates, oxides etc) while the layer constituents range widely (e.g. lipids, inorganic nanoparticles, biopolymers (proteins, polysaccharides, nucleic acids), synthetic polyelectrolytes etc. The wall can be tuned in thickness, composition and functionality by choosing various constituents and adjusting the layer number.

Preparation techniques

Precipitation method

$CaCO_3$ microparticles are usually prepared by precipitation reaction involving Ca^{2+} and CO_3^{2-} counter ions. The direct mixing of soluble salts of Ca^{2+} and CO_3^{2-} results in the formation of a nano-seed solution changing into an amorphous precipitate initially, which later converts into aggregated $CaCO_3$ micro-crystals with a particular arrangement with wide variation in size and shape. Great practical needs in $CaCO_3$ preparation consisting of uniform, homogenous size and non-aggregated microparticles stimulated many studies on controlling the crystallization process. It has been

Table 1: Excipients used in $CaCO_3$ micro/nanoparticles preparation

S/N	Category	Description	References
1	Polyelectrolytes	PSS, PAH, FITC	(Joshi and Srivastava, 2009; Stein *et al.*, 2006)
	Examples of Polyelectrolyte-labeled proteins	PSS-Rh, MW ca. 670 kDa (PSS co-polymer with Rhodamine)	(Reibetanz *et al.*, 2006)
		FITC-dextran (Mw. 4 kDa; 70, 150, 500 and 2000 kDa) PAH:dye PSS:dye	(Petrov *et al.*, 2005)
2	Proteins	Bovine serum albumin (BSA) Dextran sulfate (70, 150 and 500 kDa) Protamine	(Reibetanz *et al.*, 2006) (Joshi and Srivastava, 2009; Stein *et al.*, 2006; Petrov *et al.*, 2005)
		Bovine milk α-lactalbumin, type III, α-chymotrypsin from bovine pancreas, chicken's egg, lysozyme, N-benzyl-L-tyrosine ethyl ester (BTEE)	(Rosu *et al.*, 1998) (Joshi and Srivastava, 2009)
3	Enzymes	Horseradish peroxidase, glucose oxidase	(Joshi and Srivastava, 2009; Stein *et al.*, 2006)
4	Complexing agent	Ethylene diaminetetracetic acid (EDTA)	(Rosu *et al.*, 1998)

shown that the quality of the resultant microparticles are strongly dependent on the experimental conditions including salt type and concentration, rate of mixing the solution, intensity of agitation of the reaction mixture, pH, temperature as well as parameters affecting the nucleation rate process (Jin *et al.,* 2009; Ibarz *et al.,* 2001; 2002; Tiourina *et al.,* 2001; Knoll 1996; Ladam *et al.,* 2000; 2001; Balabushevitch *et al.,* 2003; Gao *et al.,* 2002; Tiourina and Sukhorukov, 2002; Antipov *et al.,* 2003). Any additive (e.g. divalent cations, organic solvents, pH, polynucleotides and/or macromolecules) in addition to the reaction mixture greatly affect the morphology of the $CaCO_3$ microparticles produced (Volodkin *et al.,* 2003; Sukhorukov *et al.,* 2004; Volodkin *et al.,* 2003; Dahne *et al.,* 2001). Our preliminary investigations also confirmed the effect of solvent type and storage time on the morphology of $CaCO_3$ microparticles (Fig. 1)

Therefore, colloidal crystallization from supersaturated solution of rapid mixing of equal volumes of $CaCl_2$ and Na_2CO_3 solutions produces uniform, nearly spherical microparticles of $CaCO_3$ with narrow size distribution (average diameter about 5 μm). In summary, 0.33 M Na_2CO_3 solution is rapidly poured into an equal volume of 0.33 M solution of $CaCl_2$ at room temperature, followed by intense agitation on a magnetic stirrer. This is followed by filtration and thorough washing of the precipitate with pure Milli-Q (mQ) water and then the microparticles are air dried or put in an oven at 40 °C (Petrov *et al.,* 2005) as shown in Fig. 2. However, the development of such a system having spherical, stable, non-aggregating microparticles has opened new avenues for preparation, processing and encapsulation of macromolecules.

Adsorption method

Two approaches have been employed to capture proteins or polyelectrolytes (e.g. PAH) by microparticles. First is the direct adsorption of macromolecules from solutions unto preformed $CaCO_3$ microparticles, otherwise termed physical adsorption (Fig. 3). Here, equal volumes of protein (e.g. PAH) solutions of increased concentration up to 2 mg/ml are added into tubes containing weighed quantity of dry $CaCO_3$ microparticles (15 mg), and the suspensions are thoroughly agitated on a microshaker for 2 h at room temperature. Other polyelectrolytes like FITC-labelled proteins or TRITC-labeled proteins can be used such that the difference in the intensity of fluorescence of the initial protein (PAH) solutions and the appropriate supernatants obtained after centrifugation of the suspensions will serve as a measure of the quantity of fluorescent-labeled proteins (or PAH) adsorbed. Secondly, polyelectrolytes or proteins (e.g. PAH) are captured by $CaCO_3$ microparticles in the process of their formation (co-precipitation). In co-precipitation, equal volumes of 0.33 M solution of Na_2CO_3 is added to 0.33 M $CaCl_2$ solution containing graded concentration of fluorescent-labeled proteins (or PAH) so that the growing $CaCO_3$ microparticles capture them on the process.

LBL technique

The encapsulation of bio-molecules on porous $CaCO_3$ microparticles has in turn been aided by nanoengineered layer-by-layer (LBL) self assembly, which is an electrostatic self assembly method where multiple nano-sized layers are built up on the template using oppositely charged polyelectrolytes, Fig. 3 (Decher and Schlenoff, 2003). Generally, polyelectrolyte microcapsules are prepared by

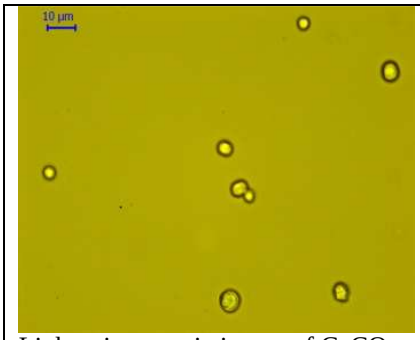
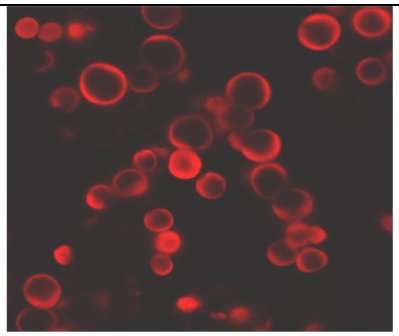
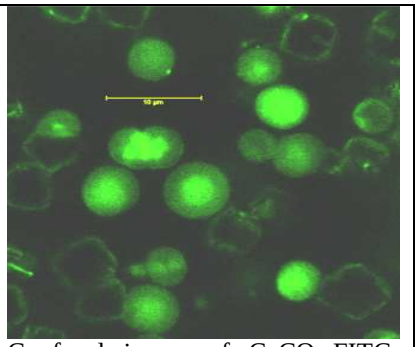
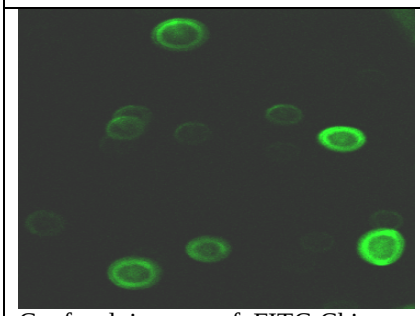
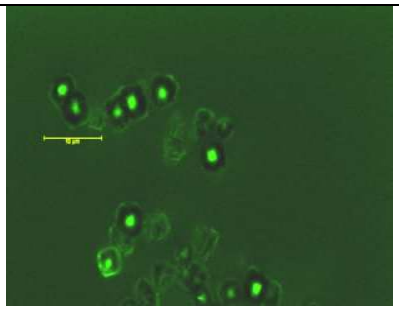
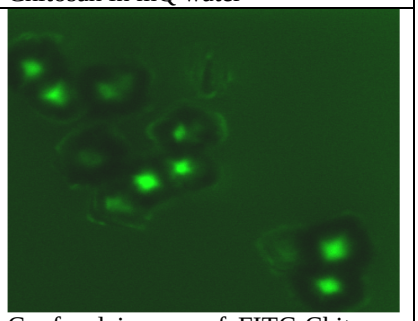
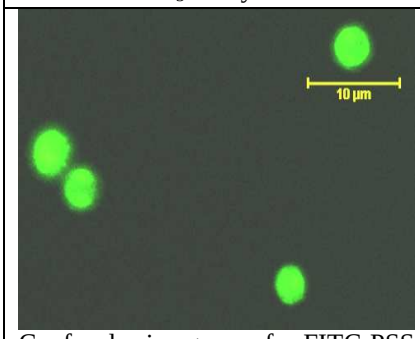
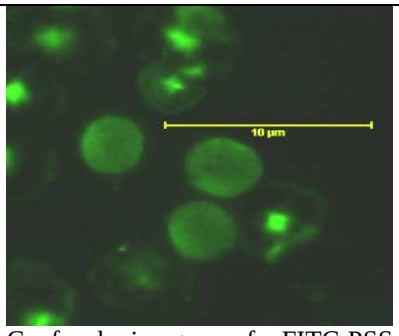
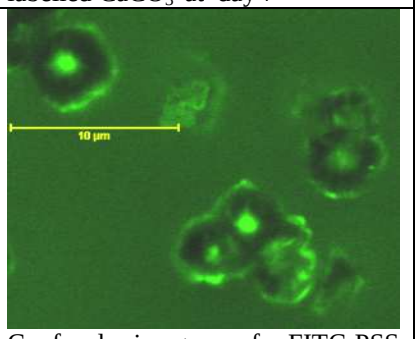
 Light microscopic image of $CaCO_3$	 Confocal image of $CaCO_3$-Rho-PSS in mQ water	 Confocal image of $CaCO_3$-FITC-Chitosan in mQ water
 Confocal images of FITC-Chitosan labelled $CaCO_3$ at day 1.	 Confocal images of FITC-Chitosan labelled $CaCO_3$ at day 2.	 Confocal images of FITC-Chitosan labelled $CaCO_3$ at day 7
 Confocal images of FITC-PSS labelled $CaCO_3$ at day 1.	 Confocal images of FITC-PSS labelled $CaCO_3$ at day 2.	 Confocal images of FITC-PSS labelled $CaCO_3$ at day 7.

Fig. 1: Effect of solvent type and storage time on the morphology of $CaCO_3$ microparticles

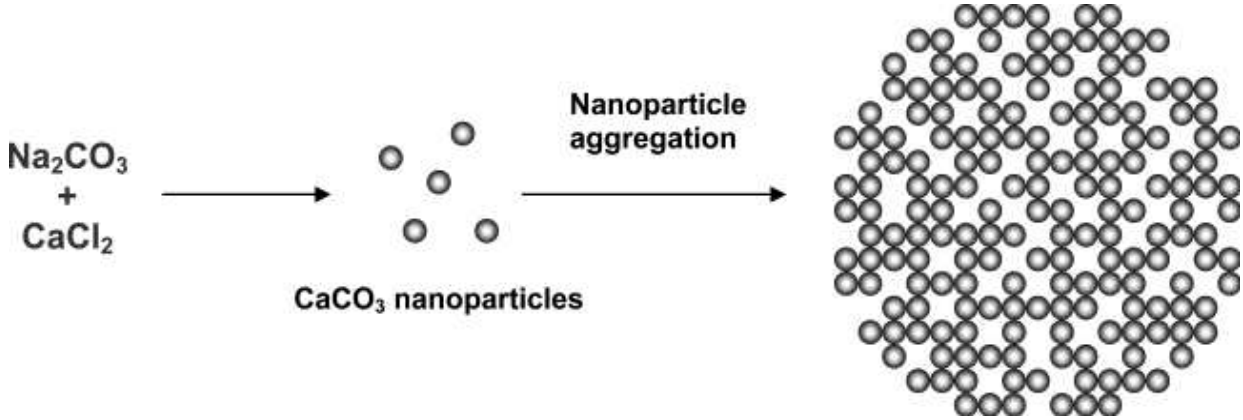


Fig. 2: Scheme of CaCO3 microparticle formation
(Modified from Volodkin *et al.*, Biomacromolecules, Vol. 5, No. 5, 2004) (Petrov *et al.*, 2005).

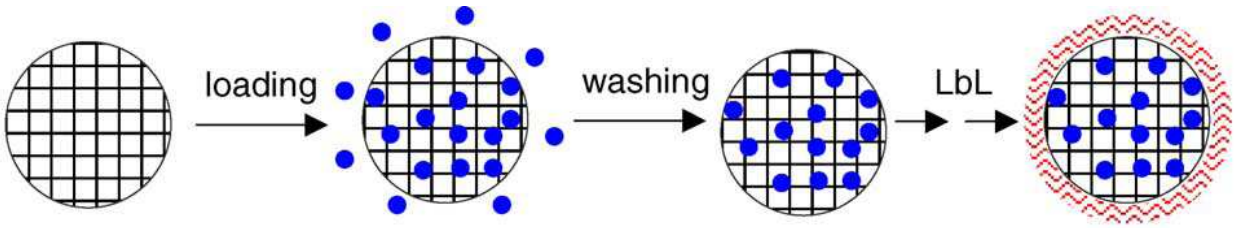


Fig. 3: Scheme of adsorption by porous $CaCO_3$ microparticles and encapsulation by polyelectrolyte multilayer assembly for drug release

(Modified from Wang *et al.*, 2006; Combination of adsorption by porous $CaCO_3$ microparticles and encapsulation by polyelectrolyte multilayer films for sustained drug delivery).

deposition of bilayers of PAH/PSS both onto pure $CaCO_3$ microparticles and unto those with proteins (or PAH) captured by physical adsorption or co-precipitation (Fig. 3). Each adsorption process of polyelectrolytes from stock solution (2 mg/ml) in 0.5 ml NaCl (pH 6.5) adjusted with HCl/NaOH involves mild agitation of the suspension $CaCO_3$ microparticles (2 % w/v) in polyelectrolyte solution on a microshaker for 15 min, centrifugation and triple washing of precipitate in 0.01 M NaCl to remove the unbound polyelectrolyte. After each centrifugation, short ultrasound pulses can be applied to the samples to prevent particle aggregation.

For LBL technique PAH is used first and PSS as outermost layer while the process of deposition can be controlled by light microscopy. The decomposition of the $CaCO_3$ core is done by multiple treatments of polyelectrolyte multilayererd-coated $CaCO_3$ particles with EDTA (0.2 M, pH 7.5) followed by washing of the microcapsules with 0.01 M NaCl and finally with water such that the microcapsules are stored as water suspensions in a refrigerator at 4 °C (Fig. 4 and 5).

$CaCO_3$ microparticles characterization

Determination of micro/nanoparticle size analysis

Scanning electron microscopy (SEM) and Optical microscopy

Both methods are used to determine particle size, particle shape and surface characteristics simultaneously. The only disadvantage lies on the fact that they can only examine small number of particles with slow and tedious distribution estimate. The diameter is obtained from two particle dimension (i.e. length and breadth) whereas particle thickness is not estimatable. Optical microscopy determines microparticulate size if distribution is monodispersed. SEM however detects submicronic particles that optical microscope cannot detect (Reibetanz *et al.*, 2006).

However, for SEM analysis, samples are prepared by applying a drop of the particle suspension to a glass slide and then drying overnight. After that, the samples are sputtered with gold and measurements are carried out (Jaspart *et al.*, 2005). A similar technology is transmission electron microscope (TEM).

Dynamic light scattering (zeta potential/electrophoretic mobility) measurement

Zeta potential is a physical property which is exhibited by any particle in suspension (Zetasizer Nano series). It can be used to optimize the formulations of suspensions and emulsions. Knowledge of the zeta potential can reduce the time needed to produce trial formulations. It is also an aid in predicting long-term stability. The liquid layer surrounding the particle exists as two parts; an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions within the boundary move it. Those ions beyond the boundary stay with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is the zeta potential. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and

LBL technique:

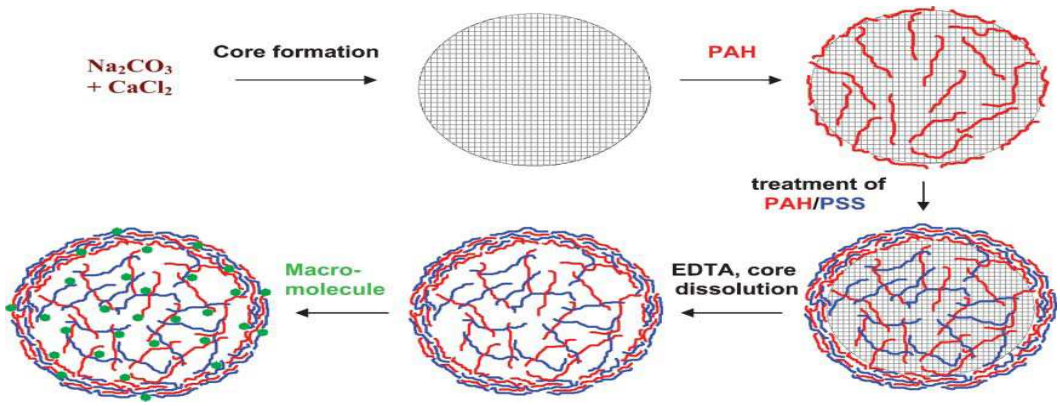


Fig. 4: Scheme of microcapsule fabrication and encapsulation of macromolecules into Capsules I (Volodkin *et al.*, 2003)

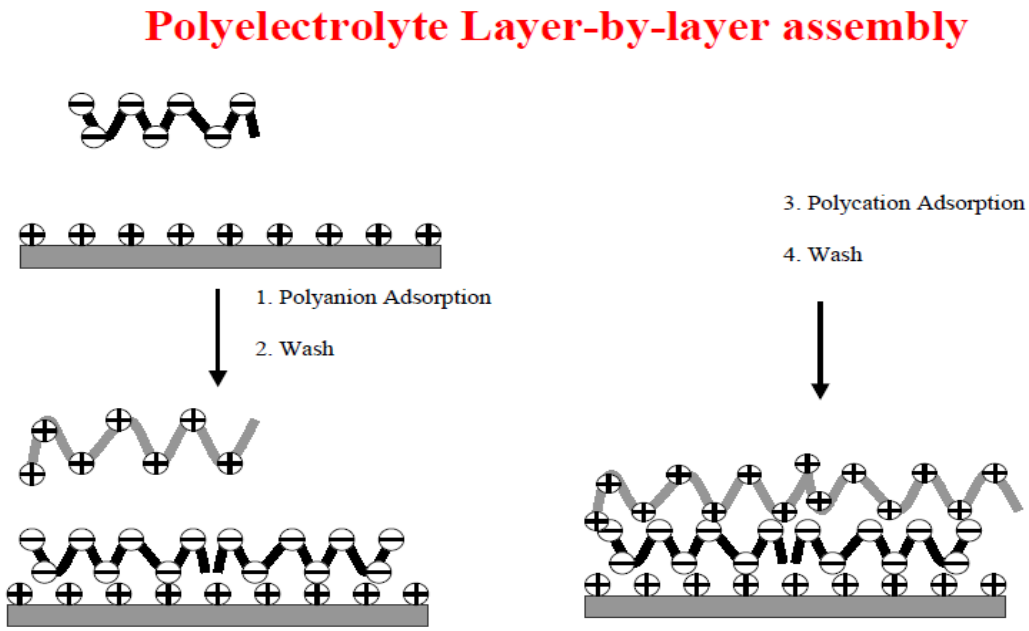


Fig. 5: Scheme of microcapsule fabrication and encapsulation of macromolecules into Capsules II (Volodkin *et al.*, 2003).

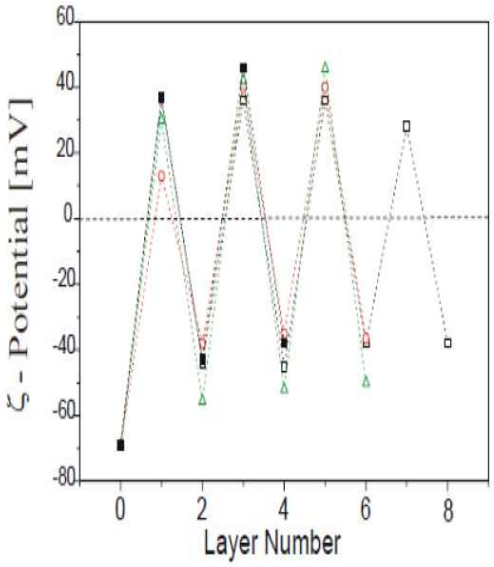


Fig. 6: Electrophoretic mobility measurement as means to follow crystal growth (Volodkin *et al.*, 2003)**.**

there will be no tendency for the particles to come together (Zetasizer Nano series). However, if the particles have low zeta potential values then there will be no force to prevent the particles coming together and flocculating. The general dividing line between stable and unstable suspensions is generally taken at either +30 or -30 mV (Fig. 6). Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable. However, if the particles have a density different from the dispersant, they will eventually sediment forming a close packed bed (i.e. a hard cake). Conductivity, pH and concentration of a formulation component are factors that affect the zeta potential measurement.

The zeta potential of the microparticles is measured after each layer adsorption by Laser light scattering. Also, zeta potential (ζ) can be calculated from the electrophoretic mobility (μ) using the Smoluchowski relation (Zetasizer Nano series)

$$\zeta = \frac{\mu\eta}{\varepsilon} \quad \ldots\ldots\ldots\ldots (1)$$

where η and ε are the viscosity and permittivity of the solvent, respectively. At a known suspension pH, the zeta potential analyzing software can be used to analyze the zeta potential measurement where the measurements are in turn reported as average value of triplicate measurements. A zeta potential versus pH curve will be positive at low pH and lower or negative at high pH. There may be a point where the plot passes through zero zeta potential. This point is called the isoelectric point and is very important from a practical consideration. It is normally the point where the colloidal system is least stable.

Classical microelectrophoresis system is a capillary cell with electrodes at either end to which a potential is applied. Particles move towards the electrode, their velocity is measured and expressed in unit field strength as their mobility. The electrophoretic mobility of the microparticles both uncoated and LBL-assembled polyelectrolyte coated microparticles are measured.

Surface area analysis

The surface area, porous volume as well as porous size distribution of the $CaCO_3$ microparticles can be determined following the Brunauer-Emmett-Teller (BET) method of nitrogen adsorption/desorption equiped with appropriate software (Reibetanz *et al.*, 2006)

Determination of $CaCO_3$ microparticle morphology

The general morphology (smooth or rough, regular or not) of $CaCO_3$ microparticle is most often determined by microscopy (SEM or optical microscopy, see 3.1.1) (Joshi and Srivastava, 2009; Reibetanz *et al.*, 2006; Petrov *et al.*, 2005; Rosu *et al.*, 1998). Their surface morphology has proved to vary depending on the excipients used (Fig. 3), as well as the form of presentation (powder or suspension) as can be seen in Fig. 4. However, the use of more recent techniques in fluorescence microscopy has made it easier to determine the shape of the microparticles.

Confocal Laser Scanning Microscopy (CLSM)/Laser Scanning Confocal Microscope (LSCM)

Confocal microscopy offers several advantages over conventional optical microscopy, including shallow depth of field, elimination of out-of-focus glare, and the ability to collect serial optical sections from thick specimens. In the biomedical sciences, a major application of confocal microscopy involves imaging either fixed or living cells and tissues that have usually been labeled with one or more fluorescent probes.

When fluorescent specimens are imaged using a conventional widefield optical microscope, secondary fluorescence emitted by the specimen that appears away from the region of interest often interferes with the resolution of those features that are in focus. This situation is especially problematic for specimens having a thickness greater than about 2 μm. The confocal imaging approach provides a marginal improvement in both axial and lateral resolution, but it is the ability of the instrument to exclude from the image the "out-of focus" flare that occurs in thick fluorescently labeled specimens, which has caused the recent explosion in popularity of the technique. Most current confocal microscopes are relatively easy to operate and have become part of the basic instrumentation of many multi-user imaging facilities. Because the resolution possible in the LSCM is somewhat better than in the conventional wide field optical microscope, but still considerably less than that of the transmission electron microscope, it has in some ways bridged the gap between the two more commonly used techniques. The confocal approach has facilitated much more useful imaging of living specimens, enabled the automated collection of three-dimensional (z-series) data, and improved the images obtained of specimens using multiple labeling.

The LSCM is currently the most widely used confocal variation for biomedical research applications ((Joshi and Srivastava, 2009; Reibetanz *et al.*, 2006; Volodkin *et al.*, 2003; Sukhorukov *et al.*, 2004). Emphasis is placed on it here since it is the design most likely to be encountered by the novice user. Other alternative designs of the instruments are favored in specific niches within the field of biological imaging. Most of the protocols for specimen preparation can be used, with minor modification, for any of the confocal instrument variants, as well as for other methodologies for producing optical sections such as deconvolution techniques and multiple-photon imaging.

In a conventional wide field microscope, the entire specimen is bathed in light from a mercury or xenon source, and the image can be viewed directly by eye or projected directly onto an image capture device or photographic film. In contrast, the method of image formation in a

confocal microscope is fundamentally different. The illumination is achieved by scanning one or more focused beams of light, usually from a laser, across the specimen. The images produced by scanning the specimen in this way are called optical sections. This terminology refers to the noninvasive method by which the instrument collects images, using focused light rather than physical means to section the specimen.

The confocal principle shows that coherent light emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector (a photomultiplier tube). As the laser is reflected by a dichromatic mirror and scanned across the specimen in a defined focal plane, secondary fluorescence emitted from points on the specimen (in the same focal plane) pass back through the dichromatic mirror and are focused as a confocal point at the detector pinhole aperture. The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not confocal with the pinhole (termed Out-of-Focus Light Rays) and forms extended Airy disks in the aperture plane (Stelzer, 2000). Because only a small fraction of the out-of-focus fluorescence emission is delivered through the pinhole aperture, most of this extraneous light is not detected by the photomultiplier and does not contribute to the resulting image. The dichromatic mirror, barrier filter, and excitation filter perform similar functions. Refocusing the objective in a confocal microscope shifts the excitation and emission points on a specimen to a new plane that becomes confocal with the pinhole apertures of the light source and detector.

However, fluorescent images of polyelectrolyte-labeled proteins of different molecular weights are examined using CLSM. Confocal micrographs of microparticle suspension dropped on a slide are taken with a flow view CLSM equipped with an inverted microscope 100x oil immersion objective lens of a known numerical aperture. The standard filter setting for fluorescence excitation is done for example at 488 nm for FITC-labeled compounds and 552 nm for Rh-labeled ones.

Scanning Force Microscopy/Atomic Force Microscopy

In scanning Force Microscopy (SFM), images are taken on a nanoscope instrument in air at room temperature using the tapping mode (Reibetanz *et al.,* 2006). A drop of the particle suspension is applied onto a freshly cleaved mica substrate followed by drying under a gentle stream of nitrogen. The image obtained on the capsule is analyzed in terms of height profile in a cross section run across the capsule. The thickness of the PAH/PSS microcapsules are determined from the height profile. Apparently, the capsule thickness can be explained by the presence of protein inside and the rough surface of the $CaCO_3$-protein microparticles used for capsule preparation (Reibetanz *et al.,* 2006).

Solid-state analysis of $CaCO_3$ microparticles

Solid state characterization is important in order to detect possible modification in the physicochemical properties of the biomolecules (e.g. drugs and enzymes) incorporated into $CaCO_3$ microparticles and of the excipients (Muller *et al.,* 2000; Reibetanz *et al.,* 2006; Müller-Goymann, 2004). The following methods are used to analyze the bulk materials as well as the solid dosage forms ($CaCO_3$ microparticles).

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is one of the most widely used techniques to study solid state, and especially to determine compound purity, stability and polymorphism (Nnamani, 2010; Nnamani, *et al.,* 2010a-d; Mehnert and Mader, 2001). This technique relies on the principle that solid-state modifications are characterized by different melting points and melting enthalpies (Mehnert and Mader, 2001). DSC therefore measures transition temperatures (solidification and melting temperatures, glass transition temperature, thermal degradation temperature) as well as transition enthalpies (Martin, 1993).

X-ray diffraction

X-ray diffraction is based on the principle that X-rays are diffracted by crystals, considering that their wavelengths have about the same magnitude as the distance between crystal atoms or molecules. This technique makes it possible to investigate a crystal structure (Trau *et al.,* 2006; Abiada *et al.,* 2010; Martin, 2004) and assesses the compound's possible amorphisation. It elucidates some polymorphic transformations and studies interactions between active substances and microparticle excipients (Joshi and Srivastava, 2009; Passerini *et al.,* 2002).

Fourier transform raman spectroscopy and infrared spectroscopy (FT-IR)

FT-IR is a useful tool for investigating the structural properties of materials. It gives the structural differences in a molecule's functional groups that can take place during crystallisation or polymorphic transformations (Joshi and Srivastava, 2009; Kaneko *et al.,* 1999; Lukeman *et al.,* 2008; Yu *et al.,* 2004). As a result, they can be used in the field of $CaCO_3$ microparticles to study the solid-states of bulk materials or solid dosage forms, and in particular to detect interactions between active substances and excipients.

Drug and macromolecule encapsulation

Generally, macromolecules have been loaded on $CaCO_3$ microparticles using different techniques viz: precipitation, co-precipitation, physical adsorption of preformed $CaCO_3$ microparticles or preformed polyelectrolyte coated microparticles (Figs. 4 and 7) of which confirmation is basically obtained using fluorescent microscopic imaging (CLSM) (Volodkin *et al.,* 2003). The fluorescent images obviously demonstrate the presence of polyelectrolyte:dye (e.g. FITC-dextran, PAH:dye, PSS:dye, Rd:dye, etc) within the micro/nanoparticle matrix. The confocal images can reveal the molecular weight data of the polyelectrolyte-protein encapsulated during preparation and washing of the $CaCO_3$ microparticles as well as the porosity of the particles (Figs. 3 and 4) (Volodkin *et al.,* 2003; Wang *et al.,* 2006).

Fluorescent spectro-photometric study of $CaCO_3$ microparticles also confirms the extent of encapsulation of dye:proteins which is indicated in supernatant solution after washing the microparticles in eppendorf tube, in comparison to blank standard solution of the dye:protein (Joshi and Srivastava, 2009; Reibetanz *et al.,* 2006; Antipov *et al.,* 2003; Sukhorukov *et al.,* 2004). Adsorption and entrapment are also indicators of encapsulation efficiency of macromolecules on preformed $CaCO_3$ microparticles. Also, UV spectrophotometric analysis showing a decrease in the intensity of supernatant solution in comparison to standard solution at λ max is also an indicator of

encapsulation in $CaCO_3$ microparticles.

The encapsulation efficiency (EE) is calculated generally from the calibration curve of the macromolecules and can be obtained as the percentage related to the total amount of macromolecule initially used (Sukhorukov *et al.*, 2004).

$$EE(\%) = \frac{amount\ of\ macromolecule\ incorporated}{amount\ of\ initial\ macromolecule} x100 \ldots\ldots\ldots (2)$$

In the case of adsorption process, the macromolecular adsorption capacity can be calculated from

$$Q = \frac{P(Ao \quad A)}{AoN} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Where Q is the amount of protein adsorbed per one $CaCO_3$ microparticle, A is the measured fluorescence, Ao is the fluorescence determined using supernatant solutions of $CaCO_3$ microparticles instead of microparticle suspension, P is the common protein content in the particle suspension, and N is the number of microparticles in the suspension (Sukhorukov *et al.*, 2004). The concentration of particles can be calculated with a bright-line hematocytometer (a cell counting chamber).

By varying the concentration of the macromolecular solutions at constant pH and adding the $CaCO_3$ microparticle suspension at the same pH into the protein solution, the adsorption isotherm can be established (Joshi and Srivastava, 2009; Sukhorukov *et al.*, 2004).

In vitro release study

A drug release study is done on prepared microparticle to determine how the incorporated drug or bio-molecule is able to release and the manner of release, whether immediate, sustained or controlled (Nnamani,

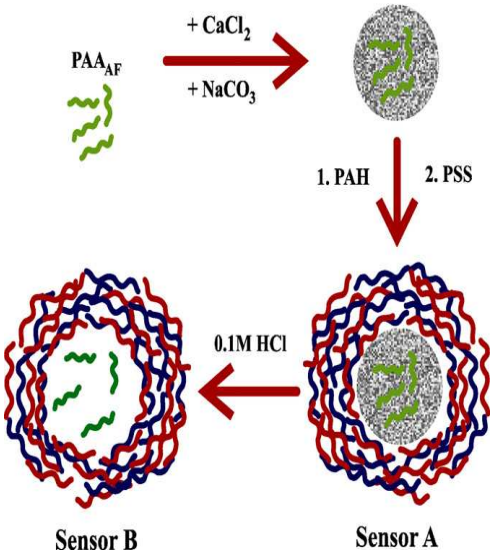


Fig. 7: Biosensor encapsulation (Volodkin *et al.*, 2003).

2010). Because of the minute nature of particles formed with $CaCO_3$ microparticle, drug release profiles determined using the conventional *in vitro* dissolution test as carried out according to the Pharmacopeia (USP or European Pharmacopeia) guidelines may not represent an ideal *in vitro* determination; for example, by using a basket or paddle stirring apparatus. Therefore, the drug-loaded $CaCO_3$ microparticles, the encapsulated drug-loaded $CaCO_3$ microparticle or the microcapsules are put in a dialysis bags with known molecular weight cut-off (MWCO) and then immersed in appropriate release medium which can be simulated gastric fluid (SGF, pH 1.2) or simulated intestinal fluid (SIF, pH 7.4), and stirred continuously at 37 °C (Volodkin *et al.*, 2003). At certain time intervals, a known volume of the release medium is withdrawn and the concentration of drug or bio-molecule is determined by UV absorbance and the solution is returned immediately after measurement. In the special case of topical administration, the drug release study may be done with the help of the Franz diffusion cell technique (Nnamani, 2010; Nnamani *et*

al., 2010d; Yener *et al.,* 2003). It has also been observed that a 24 h time interval is considered sufficient to study the sustained-release potential of drug carrier systems.

Drug release is expected to be affected by several parameters (Bodmeier *et al.,* 1992; Savolainen *et al.,* 2002; Blanco-Prieto *et al.,* 1999; Woo *et al.,* 2001; Sukhorukov, 2001; Takeshi *et al.,* 2006; Sfeir *et al.,* 2003). First, the dissolution medium and the dissolution method must be both correctly chosen in order to get a correct prediction of the *in vivo* drug release from microparticles. The choice of the dissolution medium and method can actually affect the *in vitro* drug release study results and, therefore, their correlation with the *in vivo* drug release study. The nature (hydrophobicity) of the excipient is considered to be the most important parameter influencing drug release. More hydrophobic materials are expected to reduce the drug release rate (Tiourina and Sukhorukov, 2002; Sukhorukov *et al.,* 2004). The drug's physicochemical characteristics (its water solubility) also play a part (Ladam *et al.,* 2001; Balabushevitch *et al.,* 2003; Tiourina and Sukhorukov, 2002). The release rate and the amount of drug released from $CaCO_3$ microparticles increase with drug hydrophilicity. The particle size is also considered a relevant parameter influencing drug release. Drug release from smaller particles is higher than release from larger ones because of the larger specific surface area of smaller microparticles (Nnamani, 2010). A faster release is obtained with higher drug and/or adjuvant content. The drug release increases when the medium agitation rate in the dissolution apparatus increases (Nnamani, 2010). Storage can induce polymorphic changes in $CaCO_3$ microparticles and thereby modify the drug release rate (Volodkin *et al.,* 2003). Consequently, a suitable choice of $CaCO_3$ microparticles formulation (in terms of excipient nature, drug nature and drug loading) can bring about the intended *in vitro* release profiles (e.g., sustained release, enhanced release (Joshi and Srivastava, 2009; Reibetanz *et al.,* 2006; Volodkin *et al.,* 2003; Sukhorukov *et al.,* 2004). If obtained $CaCO_3$ microparticles are not rinsed after separation from the aqueous phase, the dissolution profile shows a rapid release from the external drug fraction towards the dissolution medium, followed by a phase of decrease in the release rate (Muller *et al.;* 2000; Balabushevitch *et al.,* 2003). At the end of the release study, some of the drug may remain enclosed in the particles (Balabushevitch *et al.,* 2003), in particular if the drug is absorbed into the matrix material (Balabushevitch *et al.,* 2003).

Porous $CaCO_3$ micro/nanoparticles are also used for encapsulation of macromolecules which have been assisted by nanoengineered LBL-self assembled technique as a matrix for wide application including enzyme-based biosensors (Schüler, *et al.,* 2000) and enzyme immobilization (Schüler, *et al.,* 2000; Trau *et al.,* 2006), and has been shown to exhibit high loading efficiency and stability. Macromolecular release from uncoated and polyelectrolyte microparticles can be subjected to a time dependent release of dye:protein from the encapsulated matrix which can be monitored using fluorescence spectrophotometer by ratiometric analysis of the supernatant and standard solution. The supernatant is obtained by centrifugation of the dye:protein loaded $CaCO_3$ microparticles at about 5200 g for 10 min and fluorescence emission is acquired at an excitation wavelength for the dye (e.g. 488 nm for FITC) and compared with standard solution of labeled macromolecule using fluorescence spectrophotometer. The

ratio of fluorescence emission of supernatant and standard solution is compared against time for different batches of uncoated (bare $CaCO_3$ microparticles) one bilayer coated and two-bilayer coated microparticles to estimate the dye:protein release (Joshi and Srivastava, 2009).

Biocompatibility and application of capsules generated from $CaCO_3$ microparticles

Calcium carbonate as a component of the biological system is considered a safe material for administration into the biological system in addition to its confirmed dissolubility leaving no residue within the polyelectrolyte capsules (Fig. 8) (Trau *et al.*, 2006; Volodkin *et al.*, 2003). This leaves us to deal solely with the polyelectrolytes that constitute the capsule shell, the most widely investigated polyelectrolyte pairs being PSS and PAH. Both are regarded as bio-incompatible and hence the use of capsules made with them for biological applications is not possible. But we cannot allow this nature-given material ($CaCO_3$) that promises much in biomedical research to lay waste. Something must be done!

In order to impart biocompatibility to the polyelectrolyte capsules, several biopolymers as multilayer constituents of polyanionic polymers such as alginates, poly-glutamic acid, dextran sulfate etc, and polycationic polymers such as polyornithine, protamine and chitosan are currently exploited to re-fabricate the capsules into bio-friendly materials (Volodkin *et al.*, 2003), even though so far, polyornithine (150 kDa M.W.)-alginate/polyglutamic acid pair produces the most stable capsules but the investigations have met with strong aggregation of particles during the LbL assembly procedure which was more pronounced when chitosan was used as the polycation, apparently, suggesting that the adsorbed chitosan does not introduce enough electrostatic repulsion and hence screens the inter-particulate adhesion (Volodkin *et al.*, 2003). The use of protamine as polycation failed mainly at the stage of core dissolution, as polyelectrolyte capsules obtained with protamine with any of the polyanions listed above could not retain stability due to the limited number of positively charged groups available in protamine for electrostatic complexation. The most remarkable feature of successfully fabricated biocompatible capsules having polyornithine and polyanions was the absence of a matrix structure within the capsule interior (3 layer-pairs of polyornithine/ alginate with the first layer of polyornithine being labeled with a rhodamine derivative)

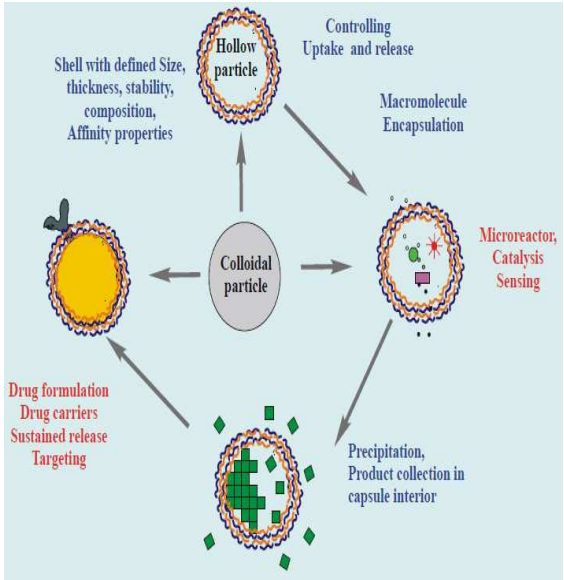


Fig. 8: Representative model for general application of $CaCO_3$ microparticles/capsules (Volodkin *et al.*, 2003).

(Fig. 9) (Volodkin *et al.*, 2003). As a result, there was no fluorescence from the interior of the capsules (as was the case with the PSS-PAH pair showing a matrix-type structure). This could be because of slow penetration of the bulkier biomacromolecules into the template pores. When the first layer of

macromolecules was still on the exterior of the CaCO3 particle (not yet diffused into the pores), there was the possibility of being complexed there by the incoming second layer of oppositely charged polyelectrolyte—thus effectively blocking any further pore-mediated diffusion (Volodkin *et al.,* 2003). However, the polyornithine/alginate acid capsules in the presence of FITC-dextran showed that the capsules were not permeable to high molecular weight dextran in a wide range of pH and salt concentration indicating the closure of pores for active loading of the substrates (Volodkin *et al.,* 2003). In any case, the $CaCO_3$ microparticles offer the feasibility to fabricate completely biocompatible capsules via template-directed LbL assembly there by opening up an opportunity to explore these capsules in their interaction with cell tissues.

Other fabrication processes are currently on-going to improve the biocompatibility of capsules for bio-molecular applications. Unlike the classical decomposable templates that function as supports for building multilayers, $CaCO_3$ cores can also serve as a source for cross-linking that hardens the deposited shell instantly during the core dissolution process (Volodkin *et al.,* 2003). Depending upon the shell wall material and the method used for its deposition, the $CaCO_3$ assisted shell reinforcement (otherwise called surface controlled precipitation, SCP) may result in formation of micro-gel-like structures (Fig. 9) taking advantage of the insolubility of sodium alginate in hydro-alcoholic solutions (approx. 30% v/v or more of ethanol) which even forms thicker shell walls (Sukhorukov, 2001; Takeshi *et al.,* 2006; Sfeir *et al.,* 2003; Ribeiro *et al.,* 2004; Dudnik *et al.,* 2001). In addition, a considerable amount of the alginate material is precipitated as a fine colloidal substance which gets entrapped within the fenestrations and channels of the highly porous template. So that when $CaCO_3$ is decomposed by hydrochloric acid, the calcium ions generated in-situ instantly crosslinks the sodium alginate present in the vicinity (by virtue of physisorption and pore diffusion) leading to formation of a micro-gel-like structure. A small quantity of external cationic dye (like rhodamine 6G) that stains the anionic polymeric coating helps in visualization of the capsular microgel structure using confocal microscopy (Volodkin *et al.,* 2003).

However, if the template dissolution involves EDTA, no microstructures would be seen implying that the capsular-gel was essentially made of a calcium-cross-linked alginate matrix which can further be characterized using high resolution SFM imaging in addition to the characteristics (surface morphology and thickness or height) of the microgel formed.

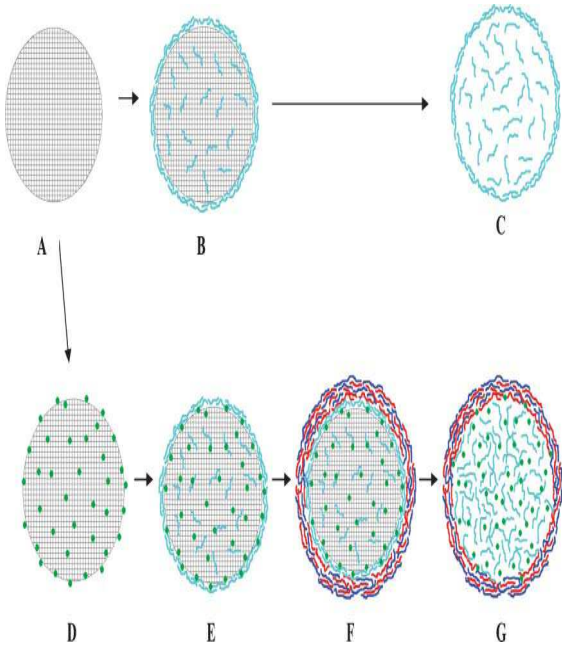


Fig. 9: Schematic representation of fabrication of microcapsules by SCP followed by template-reinforcement of the shell and fabrication of protein-filled microcapsules. (Volodkin *et al.*, 2003)

(A) $CaCO_3$ template, (B) deposition of sodium alginate (SA) by surface controlled precipitation (SCP), (C) core decomposition with HCl and in-situ crosslinking of SA by Ca^{2+}, (D) exposure to protein, (E) deposition of SA by SCP to form inner wall, (F) LbL adsorption of oppositely charged PEs to form the outer wall, (G) PE capsule filled with alginate-immobilized protein after treatment with EDTA, decomposition of the core and cross-linking of inner wall.

Great care has to be exercised not to subject such a fragile system to physical stresses like vortexing or centrifugation which dislodges the entrapped protein due to the absence of any gel-like structure. Higher amounts of alginate has been shown to contribute to the increased dry volume of the gel particles due to a considerable amount of precipitating alginate entrapped in the voids that subsequently undergo gelation when divalent calcium ions are released during the core dissolution process. Exploration of such capsular gels is difficult due to irregular cross-linking and significant interparticle adhesion (Volodkin *et al.*, 2003). Nevertheless, the method forms a basis for a simple core-assisted gel-capsule fabrication which can allow additional polyelectrolyte multilayers for possible surface stabilization, construction of a defined capsular shell wall and retention of the alginate matrix formed in-situ during subsequent treatments to derive polymer-filled capsules (Volodkin *et al.*, 2003).

The polyelectrolyte microcapsules can be loaded with biomaterials of varying interest (Joshi and Srivastava, 2009; Volodkin *et al.*, 2003; Sukhorukov *et al.*, 2004; Sukhorukov, 2001 Sfeir *et al.*, 2003). For instance, filling the capsule interior with both organic (polymers, proteins, drugs etc.) and inorganic (metal clusters, semiconductor nanocrystals etc.) materials following two basic principles - active loading (entrapment is achieved during fabrication of the microcapsules) and passive loading (encapsulation is achieved after the microcapsules are obtained and the material of interest is driven into or generated in-situ within the capsules using physicochemical forces like pH/concentration/ionic/polarity gradient, complexation, chemical reaction etc) (Volodkin *et al.*, 2003). The driving force for active encapsulation is essentially physical adsorption, pore diffusion and electrostatic interaction. Polyelectrolyte multilayers have been deposited on the adsorbed protein (e.g. Bovin serum albumin (BSA) with the intention of capturing it and the events following core dissolution (in-situ generation of a gel-like matrix) helped in immobilization and retention of the protein within the microenvironment of the capsule interior (Volodkin *et al.*, 2003). The label on the protein (e.g. rhodamine tagged to the protein) can aid in tracing the location of the protein (whether partitioned into the

core (voids) or onto the wall (adsorbed fraction) at various stages of manufacturing before EDTA treatment (Volodkin *et al.,* 2003). So far, other biocompatible polyelectrolyte pairs of recent interest are sodium alginate–chitosan and dextran sulfate–chitosan) (Jin *et al.,* 2009; Tiourina *et al.*, 2001; Balabushevitch *et al.,* 2003; Tiourina and Sukhorukov, 2002; Volodkin *et al.,* 2003; Ribeiro *et al.,* 2004).

CONCLUSION

The present work has concretely provided a current review of the multiple functionalities of porous calcium carbonate microparticles besides being merely a decomposable template for microcapsule fabrication. The principle of using the template for cross-linking and reinforcing the capsule wall assembled by SCP could be improved for various applications demanding a soft microstructure for manipulations such as drug loading with a possibility of fortifying the system by further coating by the LbL method using suitable polymers. The thickness of the polyelectrolyte is tunable with nanometric precision by the LbL technique and provides a nanoengineered diffusion barrier for encapsulated substances. The double-shell walled microcapsule provides a unique opportunity to encapsulate the substance of interest as a shell constituent or as an immobilized matrix via a decomposable inner shell which can further be exploited for targeted release of encapsulated substances (protein, drug or biosensors for imaging or certain tissue examination). The diffusion of the substance captured within the gel-like interior of the microcapsule will be governed by the properties of the microgel reservoir as well as the physicochemistry of the capsule wall constituting the diffusion barrier. Proper process optimization to ensure stability of the captured substance will make for possible application in controlled release delivery systems of bio-actives.

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