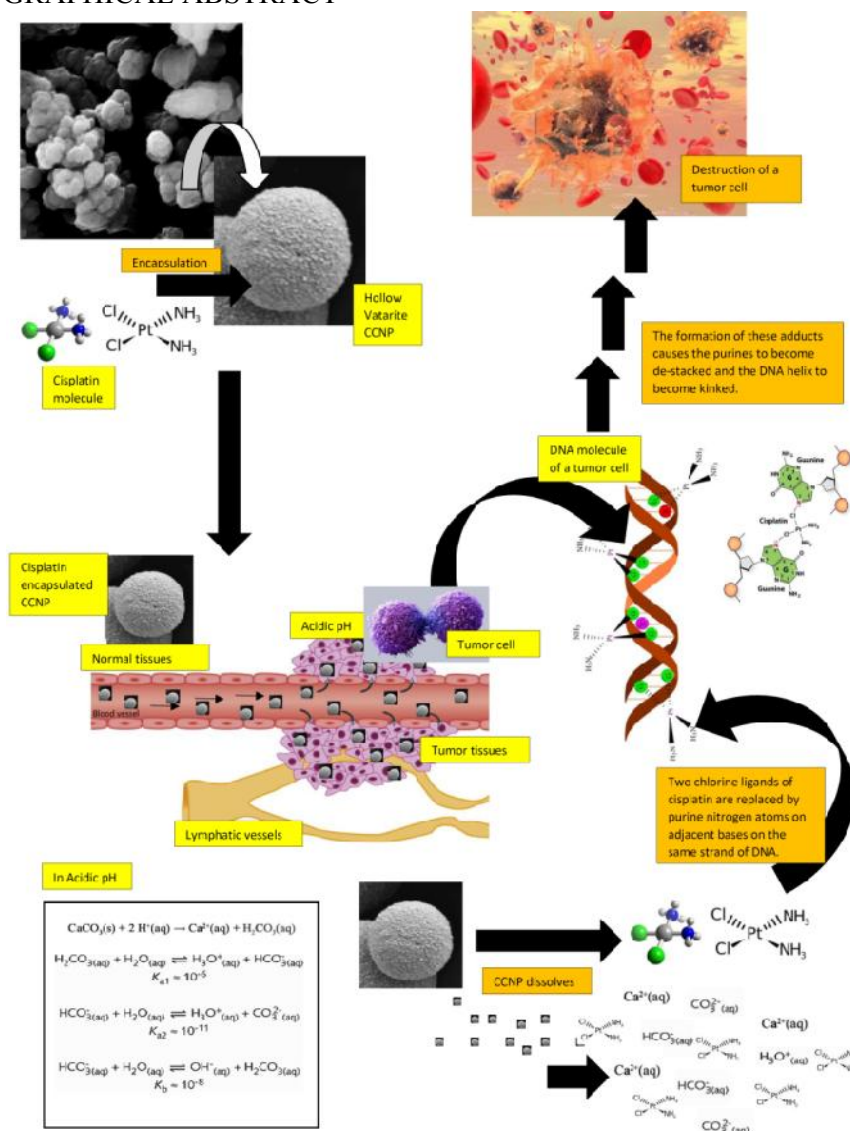


Original Research Article**SYNTHESIS OF UNSTABLE VATERITE POLYMORPH OF HOLLOW
CALCIUM CARBONATE NANOPARTICLES AND ENCAPSULATION OF
THE ANTICANCER DRUG *CIS*PLATIN****ABSTRACT**

Cisplatin is first generation platinum coordination complex-based anticancer drug developed to treat many different types of cancers. The *cis* configuration of the dichlorodiammineplatinum(II) binds to the N-atoms of the pyrimidine-purine base pairs of double or single strands of DNA and thereby cleaves DNA thus damaging the cells. As such, cisplatin is capable of not only destroying cancerous cells but also healthy normal cells making it toxic to healthy cells as well. As such, cisplatin is associated with numerous side effects. Most of these side effects and the toxicity to healthy cells can be avoided or lowered if cisplatin could be delivered only to cancerous cells in targeted manner. This will enhance the bioavailability and hence efficacy of the drug while reducing its inherent toxicity to normal healthy cells. In this research, we developed a convenient and simple wet chemical route to synthesize spherical nanoparticles of the vaterite polymorph of calcium carbonate with hollow and porous structures. Cisplatin is then encapsulated within these nanoparticles and the materials synthesized are well characterized by several independent analytical methods. The presence of cisplatin in its proper molecular form within the confined environment of vaterite is proven. Taking the advantage of the acidity of cancerous cells, these nanoparticles can be selectively and slowly dissolved only within cancerous cells thus releasing the drug only to the cancerous cells.

47 GRAPHICAL ABSTRACT



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50 INTRODUCTION

51

52 Calcium carbonate particles have widespread technological applications in building,
 53 paper, pharmaceutical, textile, rubber, plastic, paint, cosmetic, toothpaste, glove,
 54 cosmetic, food and beverage, sealing wax, ink, plastic film manufacturing and many
 55 other industries. Such a widespread use of calcium carbonate in many industries is due
 56 to several reasons which help: increasing product volume, improving processing
 57 performance such as regulating viscosity, rheological properties and curing properties,
 58 decreasing production cost, improving reinforcement properties and dimensional
 59 stability of products, improving appearance such as gloss finish, whiteness and

enhancing abrasion resistance and flame-retardant properties of products. It is an essential ingredient in the construction industry. It is used in the extraction of iron in iron industry where calcium oxide produced from heated calcium carbonate in the blast furnace produces slag with impurities. Calcium carbonate is added to drilling fluids to improve formation-bridging, filter cake-sealing and density of the fluid, to swimming pools to adjust pH of water, and is also used in refining of raw sugar. Microporous ground calcium carbonate (GCC) particles are an essential ingredient in diapers and precipitated calcium carbonate (PCC) micro particles are used in latex and nitrile-butadiene-based glove manufacturing. In medicine and healthcare products, calcium carbonate is used as inexpensive dietary calcium supplement, phosphate binder for the treatment of hyperphosphatemia in patients with renal failure and also as an inert filler of tablets and pharmaceuticals [1, 2]. It is quite astonishing to note that in some products such as plastic films, bottles etc. up to about 20% by mass is calcium carbonate and ceramic tile adhesives may contain up to 80% of calcium carbonate. In all these practical applications, if macro or micro particles that are currently used could be replaced by respective nanoparticles then the material requirement can be drastically reduced since nanoparticles have extremely large surface area to volume ratio. For instance 1 kg of $1\ \mu\text{m}^3$ particles have the same surface area as 1 mg of $1\ \text{nm}^3$ particles. Hence, in terms of surface area matching the material requirement can be reduced million-fold if $1\ \mu\text{m}^3$ particles could be replaced by $1\ \text{nm}^3$ particles, at these extreme ends.

Calcium carbonate minerals, in their pure form, such as calcite are readily available throughout the globe but calcium carbonate also exists in mixed mineral forms such as dolomite where it is approximately 1:1 CaCO_3 and MgCO_3 with SiO_2 impurities. We have designed and developed methods to separate calcium, magnesium and silica components using simple wet chemical procedures and we are currently engaged in scaling up of these processes. We have also developed procedures to prepare PCC nanoparticles and hydroxyapatite nanoparticles through bottom up approach of synthesizing them chemically from respective ions [3-12]. We then extended the procedures to develop various morphologies and phases of CaCO_3 nanoparticles, MgO nanoparticles and their nanocomposites with various polymers. However, almost all of the current synthetic methods developed to prepare calcium carbonate nanoparticles usually give either amorphous calcium carbonate or calcite since these two forms are

the thermodynamically most stable forms of calcium carbonate. However, as far as some specific applications are concerned, calcite has some drawbacks; the major one being its rhombohedral shape. Vaterite is the least stable polymorph of calcium carbonate but it is spherical in shape and its synthesis requires special techniques such as use a very specific polymers like polyaniline [8] for stabilization. In this research, we used a water-ethyleneglycol soft molecular template to prepare hollow nanoparticles of vaterite. The well characterized vaterite nanoparticles were used to encapsulate the anticancer drug cisplatin.

Cisplatin also known as cisplatinum, platamin, neoplatin, [13] with the chemical formula of *cis*-diamminedichloridoplatinum(II) (CDDP) is a first generation of platinum coordination compound-based anticancer chemotherapeutic drugs that is used to treat small cell lung cancer, ovarian cancer, lymphomas, bladder cancer, cervical cancer and germ cell tumours. It is found that cisplatin is particularly effective against testicular cancer with a cure rate of 10-85%. The *cis* configuration enables the binding of the coordination complex to two DNA strands and thereby crosslinking the DNA strands which triggers the cells to die in a programmed manner. However, cisplatin is associated with numerous side effects which include nephrotoxicity, neurotoxicity, nausea and vomiting, ototoxicity (hearing loss), electrolyte disturbance and haemolytic anaemia. The successful encapsulation of cisplatin in hollow vaterite nanoparticles described here is the first step in targeted delivery of cisplatin to cancerous cells which we are currently pursuing.

MATERIALS AND METHODS

All the chemicals except cisplatin were purchased from Sigma Aldrich and they were of analytical grade and used without further purification. Cisplatin injection bottles containing 1% cisplatin in saline water was purchased from Sri Lanka Pharmacy, Kandy, Sri Lanka.

Hollow CaCO_3 nanoparticles were synthesized by the reaction between $\text{Ca}(\text{CH}_3\text{COO})_2$ and NaHCO_3 in a solvent mixture of H_2O and ethylene glycol (EG). This method is a modification and improvement of a similar method developed by Zhao et al. [14]. Solution A is a mixture of $\text{Ca}(\text{CH}_3\text{COO})_2$ (25.0 mL, 0.50 M), H_2O (10.0 mL) and EG (25.0 mL). Solution B is a mixture of NaHCO_3 (25.0 mL, 0.50 M), H_2O (10.0 mL) and EG (25.0 mL). Then, the solutions A and B were mixed slowly in dropwise manner

under stirring. Stirring was continued for further 48 hours and the resultant suspension was filtered. The solid mass obtained was washed first with ethanol and then subsequently with distilled water and dried at 100 °C for 4 h in a vacuum oven. The CaCO_3 particles thus obtained were characterized by Laser Light Scattering based Particle Size Analysis (CILAS Particle Size Analyser NANO DS), XRD (Siemens D5000 X-ray powder diffractometer), XRF, FT-IR (Shimadzu IR-Prestige 21 Instrument with the KBr pellet method and SEM (Environmental SEM with EXAS Facilities) studies. The BET surface area was determined by the novel BET instrument constructed in our research group. In the encapsulation of cisplatin drug into the hollow CaCO_3 nanoparticle, 0.50 g of CaCO_3 nanoparticles were dispersed in a 100 mL of cisplatin injection solution and kept stirring for 24 hours to facilitate cisplatin encapsulation. The product was subsequently separated by centrifuging followed by suction filtration and washed several times with distilled water. The encapsulation of cisplatin was confirmed by the FT-IR and XRF analyses of the products obtained.

RESULTS AND DISCUSSION

As shown in Figure 1, particle size analysis shows that the colloidal solution contains particles in the nano-range between 20 nm to 80 nm with average particle size of 36.9 nm in 90% confidence interval. It is interesting to note that the wet chemical synthesis gives such a narrow distribution of non-aggregated nanoparticles of calcium carbonate which is not usually straightforward. Therefore, the soft molecular template of hydrogen bonded network of more ethylene glycol molecules and fewer water molecules form cavities of this size.

Ethylene glycol-water mixture forms hydrogen-bonded cage-like structures in which relative proportions of EG and water molecules depend upon the molar ratio of EG:water used. An extensive review of molecular templates and their applications in synthesizing supra-molecular architectures can be found in an excellent review of Hubin et al. [15]. Chen et al. have used near Infrared spectroscopy to determine molecular structures formed in pure ethylene glycol and ethylene glycol water mixtures. They found that in pure ethylene glycol only intermolecular hydrogen bonding exists and that there are no intra-molecular hydrogen bonding. As such, hydrogen bonded cage-like structures are present in pure ethylene glycol. In EG:water mixtures the relative ratio of EG:water in inter-molecular hydrogen bonded structures depend on the EG:water molar ratio used. At high EG content more EG molecules than water molecules are present in these structures forming very strong H-bonding [16].

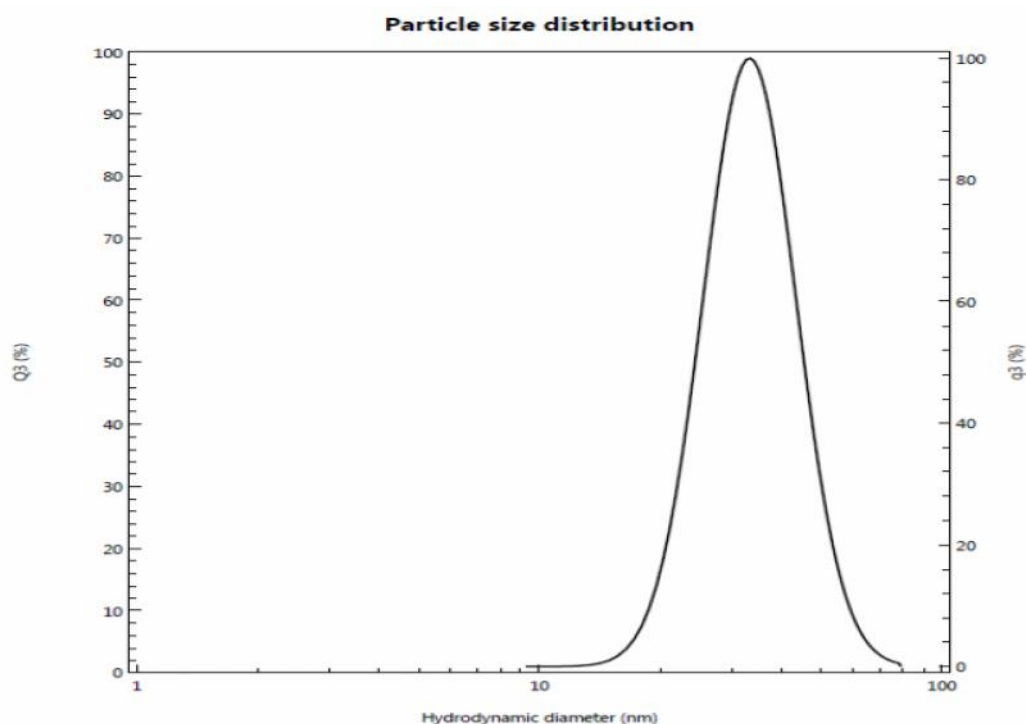
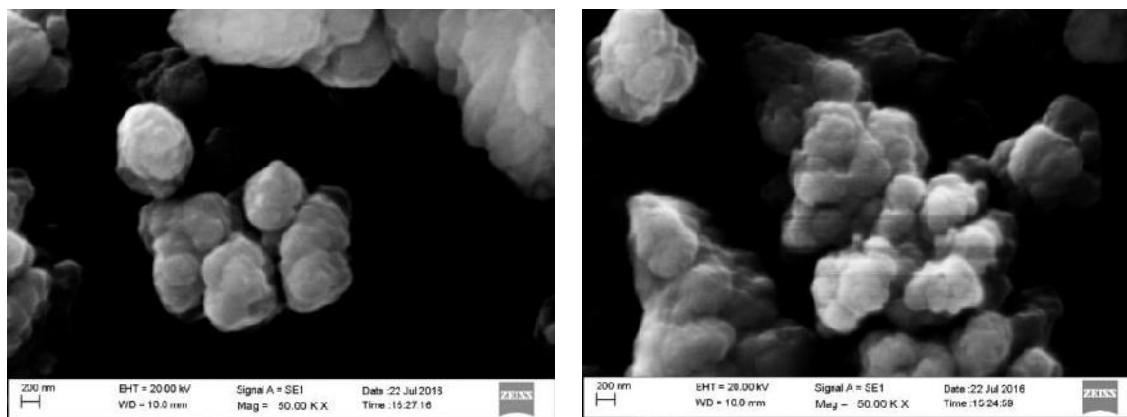


Figure 1: Particle size distribution of CaCO_3 nano-spheres.

The cavity size of these cages depends upon the number of molecules present in these cage-like structures. These cage-like structures present in EG:water mixtures can act as soft molecular templates for controlled growth of nanoparticles. Since, the cavities in these structures are mandatorily spherical in size, these soft templates can be used to synthesize spherical nanoparticles. This is the reason for the formation of spherical nanoparticles of calcium carbonate in its vaterite phase despite the fact that vaterite is not as stable as calcite in terms of thermodynamic considerations. Ethylene glycol molecules also reside inside the nanoparticles formed. Upon removal of them cavities are formed within the nanoparticles.

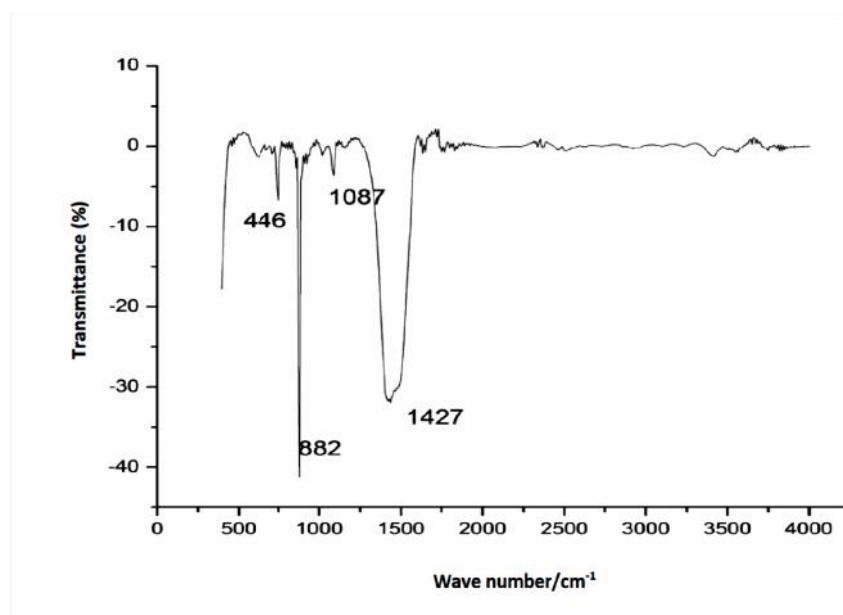
The SEM images of such hollow nanoparticles of vaterite formed are shown in Figure 2. The SEM images clearly show that the sample contains spherical particles of nano-range but clustered into aggregated particles. This clustering might have happened during deposition of particles onto glass surface for SEM analysis. As is evident from particle size determination of the suspension, discrete particles are present in the suspension. The morphology of the particles show a good porous nature. The BET surface area determination gives $1200 \text{ m}^2/\text{g}$ indicating that the particles have a huge surface area possibly due to their porous nature.

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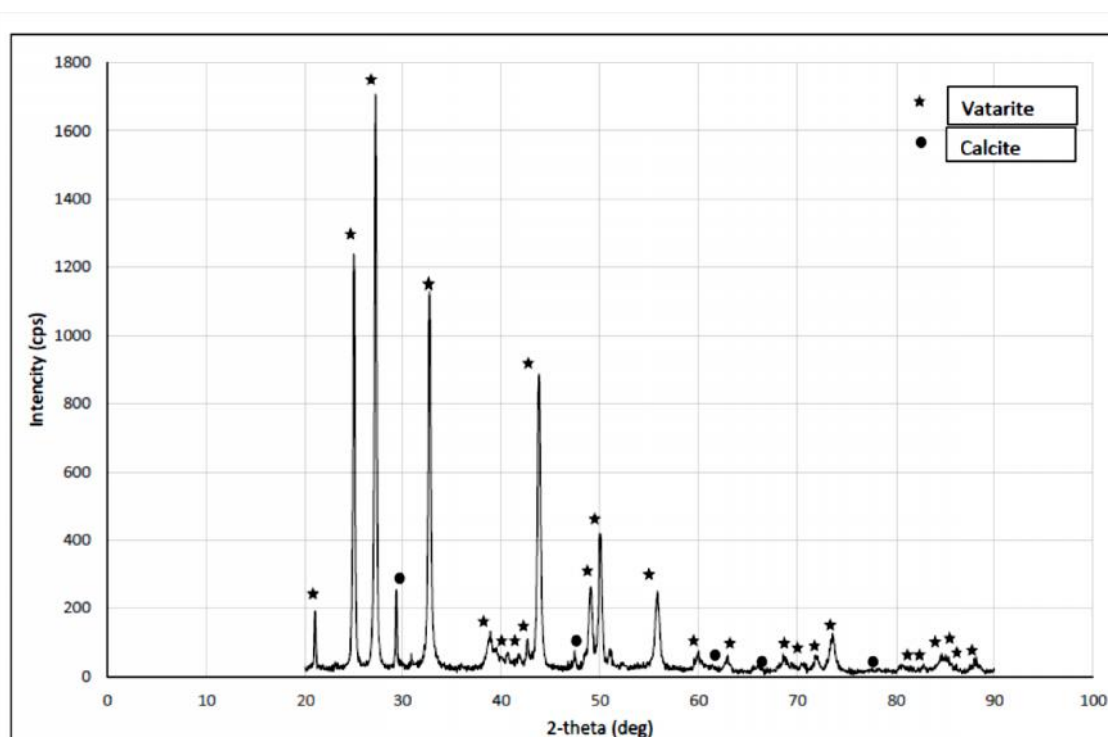
178 Figure 2: SEM image of aggregated CaCO_3 nano-spheres.

179 As evident from the FT-IR spectrum, provided in Figure 3, absorption bands at 877, 745.8
 180 and 1084 cm^{-1} confirm the presence of the vaterite polymorph of CaCO_3 . No absorption
 181 bands at $854, 712, 700\text{ cm}^{-1}$ and $848, 714\text{ cm}^{-1}$ corresponding to aragonite and calcite,
 182 respectively, are present. Since vaterite is the most unstable form of CaCO_3 , it is very
 183 interesting that our procedure produces and stabilizes this unstable vaterite form rather than
 184 forming stable calcite form. The use of molecular soft templates formed by hydrogen-bonded
 185 EG and water molecules direct the vaterite formation allowing to crystallize in spherical
 186 manner.



196 Figure 3: FT-IR spectrum of the synthesized calcium carbonate nanoparticles.

197 XRF of the CaCO_3 nanoparticles synthesized detects only Ca in our sample since our XRF
 198 machine is incapable of detecting elements with atomic number less than 13. Hence it shows
 199 that the product formed is free of impurities containing elements with atomic number higher
 200 than 13. We have further characterized the calcium carbonate nanoparticles prepared in this
 201 work using X-ray diffractometry and the X-Ray Diffractogramme obtained is given in
 202 Figure 4.

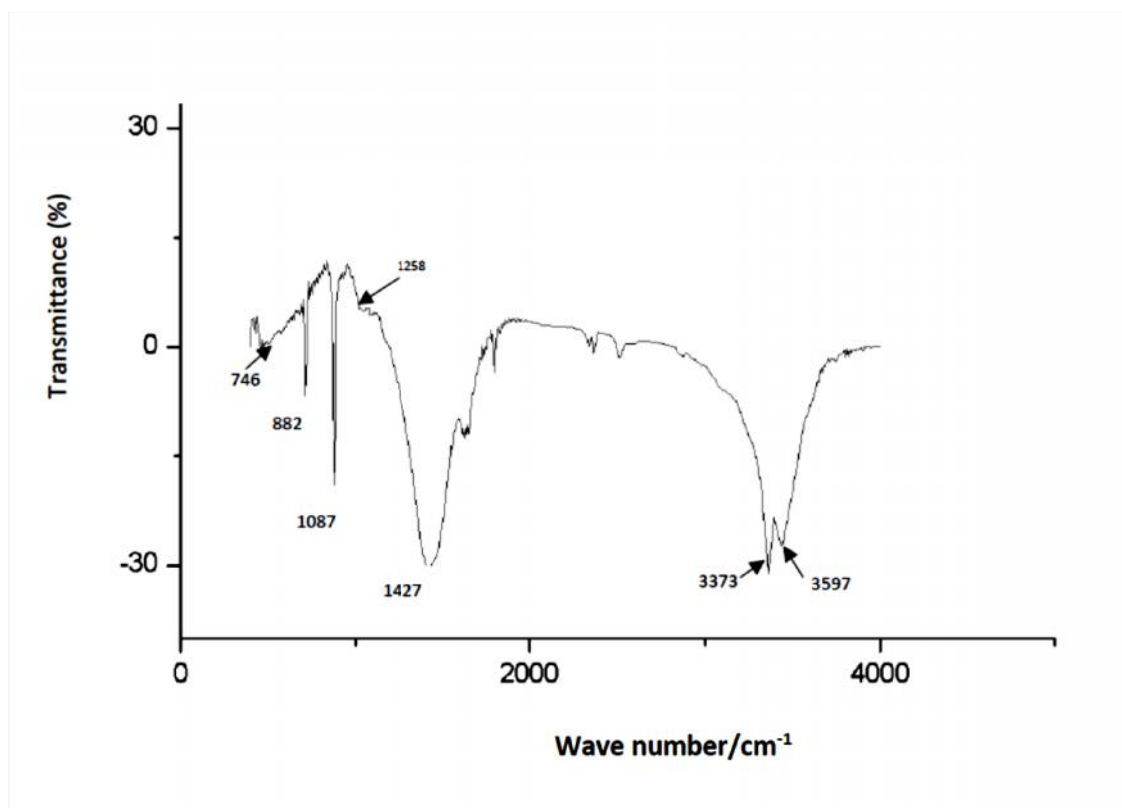


203 Figure 4: X-Ray Diffractogramme of synthesized calcium carbonate nanoparticles.

204 As can be seen from Figure 4, the sample contains basically vaterite as the major phase. A
 205 very small fraction of calcite particles can also be observed though the percentage abundance
 206 is negligible compared to that of vaterite nanoparticles. Application of the Debye-Scherrer
 207 Equation to the major XRD peak gives the crystallite size to be 28.0 nm. This is very close to
 208 the average particle size obtained by the laser light scattering based particle size analysis.
 209 This may be the reason why both FT-IR and SEM techniques do not detect the calcite. The
 210 presence of a minute fraction of calcite is not surprising since it is the thermodynamically
 211 most stable phase of calcium carbonate and the presence of these nanoparticles have no
 212 adverse effect on the encapsulation of cisplatin.

213 The encapsulation of cisplatin within these hollow nanoparticles of vaterite was performed by
 214 stirring the nanoparticles in the cisplatin injection solution (cisplatin in saline solution) as
 215 explained in the Experimental Section. The presence of cisplatin in these nanoparticles was
 216 studied through FT-IR, X-Ray Fluorescence (XRF), XRD studies and Energy Dispersive X-
 217 Ray Spectroscopy (EDX) of SEM analysis.

218 The FT-IR spectrum of the encapsulated product obtained [Figure 5] clearly show the
 219 presence of N-H antisymmetric and symmetric stretching vibration 3373 cm^{-1} & 3597 cm^{-1} ,
 220 N-H wagging together with CO_3^{2-} vibrations ,deformation vibrations (scissoring) 1650 cm^{-1} ,
 221 1590 cm^{-1} and wagging oscillation broad transmittance band 800 cm^{-1} . It also contains all the
 222 bands that are reported for hollow nanoparticles of CaCO_3 . In the FTIR spectrum, the
 223 absorption bands at 871 cm^{-1} show the presence of vaterite polymorph. As such, the FT-IR
 224 data suggest the encapsulation of cisplatin in hollow CaCO_3 .



225 Figure 5: FT-IR spectrum of cisplatin encapsulated CaCO_3 nanoparticles.

226 The XRF spectrum of the cisplatin encapsulated CaCO_3 product shown in Figure 6 clearly
 227 elicits the presence of Ca, Pt and Cl with the Pt:Cl atomic ratio of 1:2.5 showing that cisplatin
 228 maintains its identity in the confined environment also. Ideally, the ratio should be 1:2 but the

presence of excess chloride in saline solution would encapsulate free chloride ions also thus increasing the chloride content. XRF data suggest the encapsulation of cisplatin in hollow CaCO_3 .

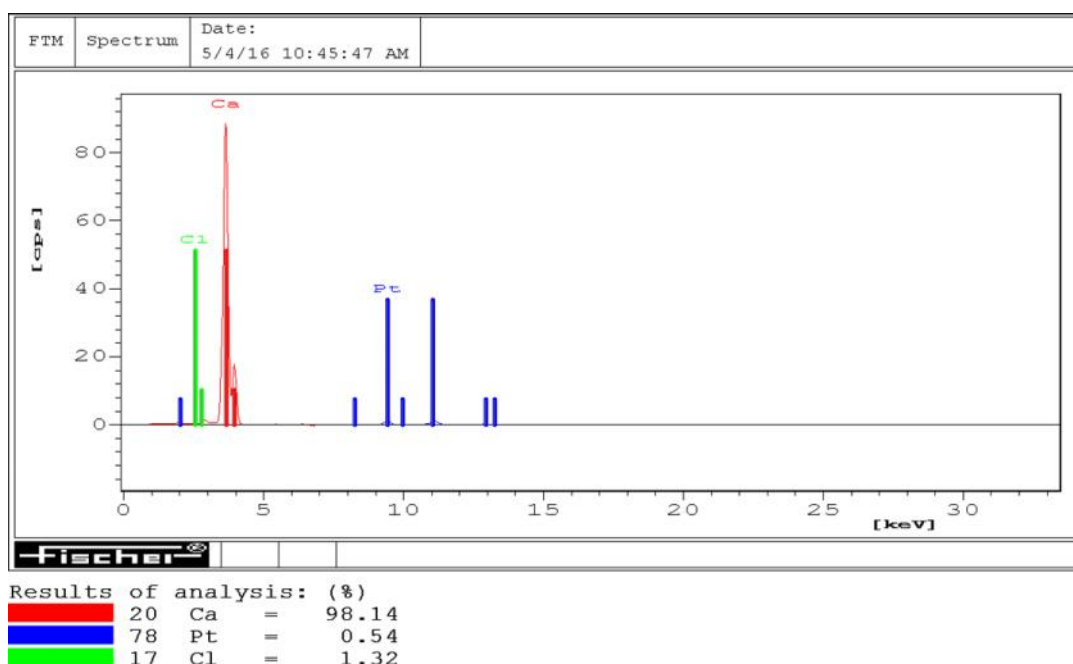
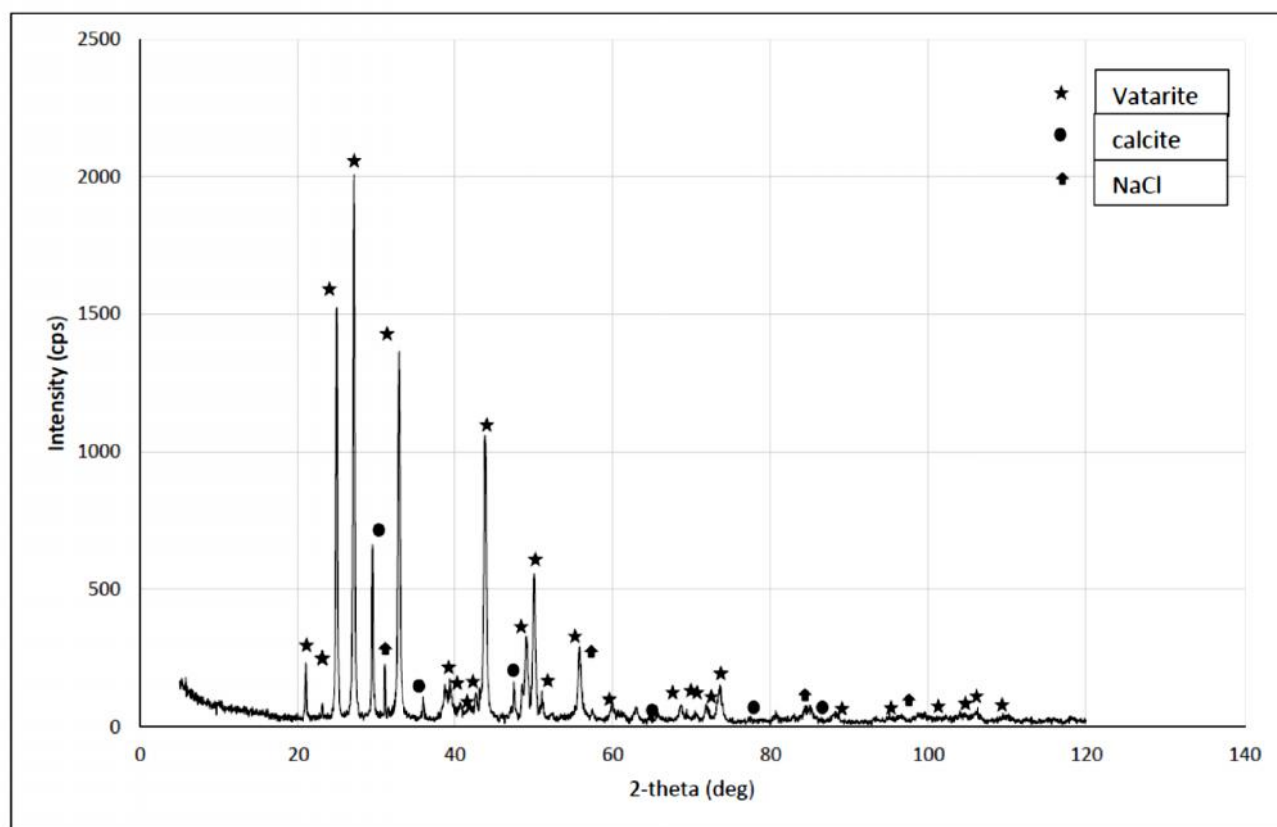


Figure 6: XRF spectra of cisplatin encapsulated CaCO_3 nanoparticles.

The X-Ray Diffractogramme of the cisplatin encapsulated vaterite nanoparticles is shown in Figure 7. It also contains vaterite as the major component with minute fractions of calcite and sodium chloride. The presence of cisplatin cannot be determined from the XRD since cisplatin is present in molecular form within the confined environment of pores of vaterite nanoparticles in non-crystalline form. However, FT-IR and XRF studies complement this showing the presence of cisplatin in encapsulated nanoparticles. The encapsulation has not affected the crystallinity and crystallite size of vaterite nanoparticles. The crystallite size calculated using the major XRD peak is 29.2 nm which is same as that without cisplatin encapsulation. Hence cisplatin molecules occupy the free spaces of hollow nanoparticles of vaterite without changing the particle dimensions. The SEM images of the encapsulated product are shown in Figure 8.

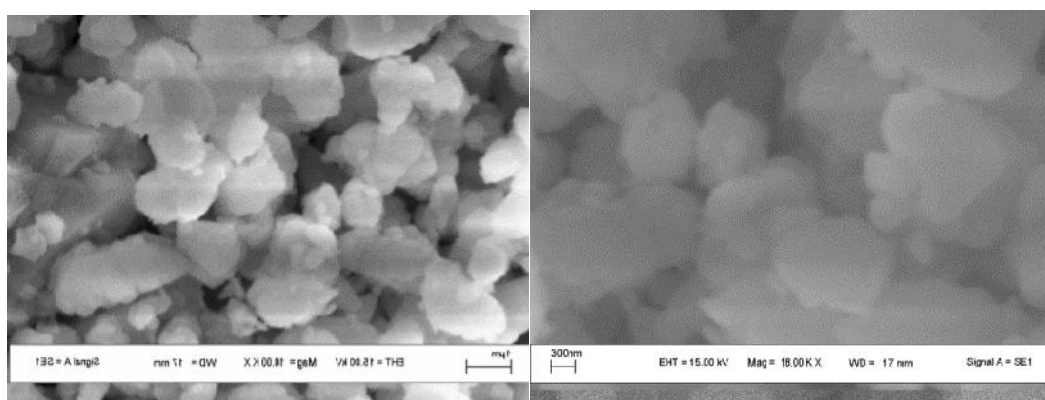
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249 Figure 7: The XRD of cisplatin encapsulated vaterite nanoparticles.

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252 Figure 8: SEM images of cisplatin encapsulated vaterite nanoparticles.

The SEM images are quite similar to those obtained for vaterite nanoparticles without cisplatin encapsulation. This shows that vaterite form of calcium carbonate is preserved even after the cisplatin encapsulation. It is interesting to note that the EDAX analysis does not show Pt, Cl or N in the cisplatin encapsulated vaterite nanoparticles though XRF clearly shows the presence of Pt and Cl and FT-IR gives evidence to the presence of N. Both XRF and FT-IR are bulk analytical techniques that measure the composition of the bulk sample. However, SEM analysis is a surface analytical technique detecting a few micrometer width from the surface and therefore EDAX detects the elements present close to the surface of the vaterite nanoparticles. Since the elements of cisplatin cannot be detected within this depth of the surface and bulk techniques detect them it can be concluded that cisplatin is present deep inside the particles at least deeper than 1 μm width from the surface. It is also intriguing that both the XRD and the light scattering based particle size analysis show 29 nm particle size the SEM show micrometer size particles. This is because in the absence of any protection these small nanoparticles attract to each other and form aggregates. When a large number of these nanoparticles assemble into aggregates cavities or hollow volumes are formed. These cavities act as hosts to reside cisplatin molecules. This is a clear evidence to prove that cisplatin is encapsulated in the centres of nanoparticles.

CONCLUSION

In this work, we developed a facile method for the synthesis of hollow spherical nanoparticles using precipitation reaction of NaHCO_3 with $\text{Ca}(\text{CH}_3\text{COO})_2$ in $\text{H}_2\text{O}/\text{EG}$ media. The colloidal solution prepared contain three sizes of particles in the nano-range with very narrow particle size distribution. These hollow nanoparticles were found to successfully encapsulate cisplatin molecules. Cisplatin retains its chemical identity within the confined environment.

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