FINAL PROGRESS REPORT OF THE WORK DONE ON THE MAJOR RESEARCH PROJECT (01-04-2013 to 31-03-2017)

Investigations on pathological deposition of biominerals

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By

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Final Progress Report:

"Investigations on pathological deposition of biominerals"

Objectives:

To study the in vitro crystallization of compounds (calcium phosphates) which cause crystal deposition diseases and to identify the factors, which inhibit and promote the aggregation and crystallization. Determining the condition in which the crystals already formed could be dissolved, modified or inhibited from the viewpoint of devising new drugs and treatment to control the crystal deposition, so that they could be treated without the surgical removal of the stones. To analyze the crystalline constituents and their relative abundance of the various stones removed from patients.

1. Mineralization of Calcium phosphates *

Calcium phosphates (CaP) are the main component of pathological deposits. They are often found in urinary stones, kidney stones and atherosclerotic plaques. They are responsible for the formation of crystal deposition diseases. The formation of such crystals depends on many factors such as food, lifestyle etc. CaP has different phases with varying Ca/P molar ratio ranging from 0.5 to 2.0. Hydroxyapatite (HAp) $[Ca_{10} (PO_4)_6 (OH)_2]$, Di-calcium phosphate dihydrate (DCPD) $[CaPO_3 (OH) 2H_2O]$ and tri-calcium phosphate (TCP) are some of the phases that play a vital role in human metabolism. Among these, HAp is the most stable phase, DCPD is comparatively less stable and these phases are mainly found to exist in pathological stones. The nucleation of such crystals occurs when the calcium and phosphate ions get supersaturated. Subsequently, the growth process depends on the temperature, pressure, etc. The first step is to grow the CaP crystals in different environments to understand the factors influencing their nucleation and growth.

Studies revealed that metal ions such as iron and cobalt present in the reactant solution can influence the morphology and also the growth rate [1]. In this project CaP crystals were mineralized in silica gel medium.

*Part of the research has been published in Materials Chemistry and Physics, 218 (2018) 166–171.

The effect of metal ions such as iron and cobalt on mineralization has been studied. The effect of magnetic field on the mineralization of iron doped CaP has also been investigated. Gel method was adapted to study the mineralization of CaP because it acts as an ideal medium to study crystal deposition diseases, which could lead to better understanding of their etiology [2]. The experiment was carried out at physiological temperature (37 ± 0.5 °C).



Figure. 1.1: Schematic representation of various phases of calcium phosphates grown in silica gel (a) without magnetic field (b) with magnetic field

The changes in morphology of the obtained crystals were studied using Scanning Electron Microscope. Their structure and roughness were also studied by X-ray diffraction technique, Infrared, Raman spectroscopy and Atomic Force Microscopy.

1.1 Optical Microscopy

Pristine platy DCPD crystals were mineralized in silica gel medium (fig 1.2 a). Iron and cobalt doped DCPD crystals are shown in (fig 1.2 b & c) respectively. The morphology of the iron doped crystals were platy and cobalt doped crystals were irregular rods. Iron and cobalt doped crystals are relatively bigger in size when compared to undoped DCPD and less number of crystals were formed with a faster growth rate.



Figure. 1.2: Optical micrograph of DCPD crystals grown in silica gel (a) pristine (b) iron doped (c) cobalt doped and (d) iron doped in the presence of magnetic field

The presence of cobalt yields more number of crystals compared to the presence of iron in the gel medium. The iron doped DCPD crystal grown under the influence of magnetic field has dendritic morphology (fig.1.2 d).

1.2 Powder X-Ray diffraction

Powder X-ray diffraction patterns of liesegang rings (fig. 1.3 a & b) and the crystals (fig. 1.3 c & d) are well matched with hydroxyapatite and DCPD respectively. There was no significant change in the morphology of HAp on application of magnetic field. In the presence of magnetic field, the intensity of major plane (020) of DCPD is decreased and the intensity of (170) plane is increased, indicating the oriented growth of crystals. In the absence of magnetic field the crystals are platy and are seen to preferentially grow along a particular direction, whereas in the presence of magnetic field stimulated growth of DCPD in all directions is observed. This has been evidenced from the change in lattice parameter along the c-axis (Table 1.1). This is due to the continuous phase transformation until reaching a stable phase.

S.No	Sample codes	a (Å)	b (Å)	c (Å)
		±0.001	±0.001	±0.001
1	HAp (without magnetic field)	9.199	9.199	6.801
2	HAp (with magnetic field)	9.228	9.228	6.829
3	DCPD (without magnetic field)	6.498	15.004	8.600
4	DCPD (with magnetic field)	6.387	15.144	5.842

 Table 1.1: Lattice parameter of DCPD crystals



Figure. 1.3: Powder X-ray diffraction pattern of (a & b) HAp and (c & d) DCPD without and with magnetic field respectively

1.3 FTIR and Raman spectroscopy

FTIR spectra showed the presence of functional groups corresponding to DCPD. In the presence of magnetic field, the bands of DCPD crystals are significantly suppressed due to the

structural change. The obtained structural changes correlate well with the structural changes seen in the XRD pattern.



Figure 1.4: Raman spectra of DCPD (a) without magnetic field (b) with magnetic field

Raman spectra of the crystals are shown in figure 1.4. Raman spectra showed two additional bands at 874 cm⁻¹ and 1055 cm⁻¹ corresponding to HPO_4^{2-} in the absence of magnetic field. These bands were absent in the case of crystals grown under the influence of magnetic field. The presence of magnetic field led to the growth of crystals which contain only PO_4^{3-} (i.e.) the crystals are devoid of HPO_4^{2-} phase.

1.4 Scanning Electron Microscopy and Atomic force Microscopy



Figure. 1.5: Scanning Electron Micrograph of the surface of DCPD (a) without magnetic field (b) with magnetic field and Leisegang rings of HAp (c) without magnetic field (d) with magnetic field

Scanning electron micrograph of the crystals are shown in figure 1.5. HAp with Plate-like morphology (fig. 1.5 c & d) was observed in the case of crystals grown under the absence of magnetic field whereas, needle like morphology was observed in the case of crystals grown under the influence of magnetic field. The DCPD crystals showed oblated triangular etch pits and hillocks on the surface.



Figure. 1.6: Atomic Force Micrograph of the surface of DCPD (a) without magnetic field (b) with magnetic field

The atomic force micrograph of the crystals are shown in figure 1.6. The roughness of the crystals grown in the absence and presence of magnetic field is found to be 35 nm and 512 nm respectively. An increase in surface roughness of the crystal in the presence of magnetic field is also observed in the SEM analysis, which could be due to the enhancement in the diffusion process.

1.5 Conclusion

Calcium phosphate crystals were mineralization in silica gel. The effect of metal ions such as iron and cobalt were studied and the effect of iron in the presence of magnetic field (0.1T) has also been investigated. Magnetic field changes the morphology of the crystals from platy to dendrites. It increases the stability of the crystals and also limits the incorporation of iron. On applying magnetic field the crystals formed contain PO_4^{3-} while, crystals grown without magnetic field contain HPO_4^{2-} . The dissolution properties of DCPD crystals depends on the hydrogen in its lattice, hence the absence of hydrogen in the case of DCPD grown under the influence of magnetic field is more stable. Magnetic field also enhances the roughness of the crystals.

2. Chemical and structural analysis of gallstones from the Indian subcontinent *

Gallstones are either cholesterol, pigment or of mixed type. The composition and characteristics of gallstones vary in different parts of the world, with cholesterol being dominant in the western hemisphere and pigment/mixed type being dominant in South Asian countries, with a shift to cholesterol type in these countries. Wide variations in the composition of gallstones have also been reported for the Indian subcontinent. [3-6].

In this project, we have collected gallstones from 20 patients suffering from gallstone disease and undergoing cholecystectomy, to represent the northern part of India, and 37 samples from Tamil Nadu to represent South India. The age of the patients was between 28 and 58 years. The stones were rinsed several times with deionized water and dried at room temperature. The samples were labeled as mixed gallstones, pigment gallstones and cholesterol gallstones. Random gallstone samples 2 each from Tamil Nadu and 3 from Uttar Pradesh were analyzed for structural and chemical characteristics.

The selected gallstones were photographed and examined under an optical stereo microscope to study the surface morphology (Fig. 2.1a-e). The south Indian gallstones were coded as G1 and G2. G1 (Fig. 2.1a) was dark brown with a well-defined rough outer surface. The G2 stone (Fig. 2.1b) was black, irregular in shape and was harder than the other gallstones. The north Indian gallstones were coded as G3, G4 and G5. The G3 was yellowish-brown with a very smooth surface whereas, the G4 was greenish-yellow with a rough outer surface. G5 was pale yellow, faceted with a well-defined smooth surface.



Figure. 2.1. Optical micrographs of the gallstones: (a and b): South Indian gallstones (G1 andG2) and (c, d and e): North Indian gallstones (G3, G4 and G5).

*Part of the research work has been published in Materials Science and Engineering C,78 (2017) 878–885.

2.1 Results and discussion

2.1.1 XRD Analysis:



Figure. 2.2. Powder XRD patterns of G1, G3, G4 and G5.

The XRD patterns of G1, G3, G4 and G5 were in good agreement with the standard patterns of cholesterol which correspond to the triclinic space group P1 (fig 2.2). The XRD pattern of G1 corresponds to that of cholesterol monohydrate. G2 did not exhibit any diffraction due to its amorphous nature. The XRD patterns of the mixed gallstones G3 and G4 indicated the presence of both anhydrous and monohydrated cholesterol, with the prominent peak of cholesterol monohydrate at 5.3° along with other characteristic peaks at 14.7° and 14.9°. The peaks corresponding to anhydrous cholesterol were observed at 12.9° and 14.0°. The XRD patterns of G5 showed peaks corresponding to anhydrous cholesterol. Generally, the gallstones were formed due to the supersaturation of unconjugated bilirubin and other bile salts.

2.1.2 IR Analysis

The samples G1, G3, G4 and G5 showed the characteristic peaks of cholesterol. The FTIR analysis revealed the presence of calcium bilirubinate in the pigment stone (G2) and moreover; carbonate and phosphate occurred in all the samples. FTIR spectroscopy provided deeper comprehension of individual molecules in gallstones. G1, G3, G4 and G5 showed the characteristic peaks of cholesterol. Suzuki and Toyoda reported that the infrared absorption spectra of calcium bilirubinate and bilirubin of the gallstone. G2, the pigment stone exhibited the prominent characteristic peaks of calcium bilirubinate. The presence of the bilirubin in the black pigment stone (G2) was confirmed by the peak corresponding to C=O stretching mode of HO=C-N group of bilirubin at 1244 cm⁻¹.



Figure. 2. 3. IR spectra of gallstones samples (a) G1, (b) G2, (c) G3, (d) G4 and (e) G5.

Kleiner et al. reported the varying amount of bilirubin in the black pigment stones. The carbonate and phosphate groups were identified in all the samples. Kothai et al. have also reported the presence of carbonate and phosphates in the gallstone. The bands due to the bendingmode of the phosphate groups were observed at 563 cm⁻¹ and 605 cm⁻¹ whereas, the bands at 962 cm⁻¹ and 1444 cm⁻¹ were due to the stretching mode of the phosphate and C=O stretching of carbonate groups respectively.

2.1.3 Scanning Electron Microscopy

SEM micrographs of gallstones are shown in figure.2.4. The plate-like crystals and needlelike crystals represent the morphology of cholesterol monohydrate and anhydrous cholesterol, respectively. The samples G1, G3 and G4 contained platy and needle shaped crystals. In G1, the length of needles is $8\pm 2 \mu m$. The plate-like crystals were found to be large in quantity compared to the needle type crystals and their length and breadth were $3\pm 1 \mu m$ and $0.8\pm 0.1 \mu m$, respectively. The G2 exhibited both globular and irregular flake like morphology formed by the lateral growth. The G3 consisted of platy crystals having an average length ($4\pm 0.8 \mu m$) and breadth ($1\pm 0.4 \mu m$) and the needle like crystals of average length ($5\pm 1 \mu m$). The G4 samples are made up of platy crystals with length and breadth of $6.0\pm 0.2 \mu m$ and $2.0\pm 0.1 \mu m$ respectively. The platy crystals were clustered together to form the bulk structure. The needles were found in very small quantity with the average length ($3\pm 0.7 \mu m$). The G5 consisted of flake like crystals ($15\pm 5 \mu m \times 5\pm 4 \mu m$).



Figure. 2.4. SEM micrographs of gallstones (a) lower magnification of G1, (b) higher magnification of G1, (c) lower magnification of G2, (d) higher magnification of G2, (e) lower magnification of G3, (f) higher magnification of G3, (g) lower magnification of G4, (h) higher magnification of G5 and (j) higher magnification of G5.

2.1.4 EDX Analysis

The EDX elemental concentrations are given in Table 2. Carbon, oxygen and traces of calcium were detected in all types of gallstones. The bilirubin pigment has four nitrogen atoms in its structure and thus, higher nitrogen content was found in G2 in comparison to the other samples. The low carbon content in G2 indicated a smaller amount of cholesterol in the pigment stones.

2.1.5 CHNS Analysis

The content of carbon, hydrogen, nitrogen and sulfur in the samples was obtained by elemental analysis (CHNS). This analysis was supported by the EDX data. Usually, the core of the pigment stones contains sulfur and large amounts of calcium and phosphorus; hence, the sulfur content was found only in G2. The nitrogen content was eight times higher in G2 compared to that of other gallstones due to the degraded tetrapyrrolic bile pigments derived from bilirubinates (biliary infection occur in gallbladder and bile ducts) and bilirubin (excreted in bile and urine). The cholesterol was very low in pigment stones (G2), and thus the carbon content was low. In all cases, traces of Mg and Na were found by AAS (always below 0.2 wt%). The G3 was not available in sufficient quantity for CHNS elemental analysis.

Sample	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulfur (%)	Calcium by
					AAS(%)
G1	77.8 ± 0.1	11.1 ± 0.2	0.76 ± 0.02	-	0.31
G2	50.5 ± 0.2	6.3 ± 0.2	8.90 ± 0.09	1.48 ± 0.04	1.90
G3	-	-	-	-	0.86
G4	80.0 ± 0.2	11.6 ± 0.1	0.10 ± 0.01	-	0.22
G5	75.6 ± 0.6	10.3 ± 0.4	0.63 ± 0.04	-	0.54

Table 2.1 : Elemental analysis of gallstones G1, G2, G3, G4 and G5.

2.1.6 ¹³C NMR and ¹H NMR spectroscopy



Figure. 2.5. ¹³C NMR spectra of (a) G1, (b) G3, (c) G4 and (d) G5.



Figure. 2.6. ¹³C NMR spectra of (a) G1, (b) G3, (c) G4 and (d) G5.

¹³C and ¹H NMR spectra of the CDCl3 extracts of all gallstones showed Cholesterol was in all the samples except in G2, where no soluble species were found (no NMR signals). No water molecules could be detected in G2 and G5 whereas it could be detected in G1, G3 and G4, by 1H NMR spectroscopy.

2.2 Conclusion

Gallstones were collected from the northern and southern part of India. The elements present in the gallstones were identified by physicochemical characterizations such as XRD, FT-IR, NMR, CHNX and EDX. The constituent crystals of south Indian gallstones were globular, whereas the north Indian gallstones contained platy crystals of cholestrol. The pigment stone was confirmed to be amorphous and calcium bilirubinate was identified as one of the constituents by FTIR spectroscopy. In addition, cholesterol monohydrate and cholesterol anhydrous were confirmed in north Indian gallstones by XRD analysis. EDX spectroscopy revealed the presence of silicon only in north Indian gallstones. The predominant constituents of south Indian gallstones were cholesterol and calcium bilirubinate confirmed by IR spectroscopy. However, carbon, nitrogen, oxygen, calcium, sulphur, sodium and magnesium and chloride were present in all stones.

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List of papers published

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Impact of magnetic field on the mineralization of iron doped calcium phosphates



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HIGHLIGHTS

GRAPHICAL ABSTRACT

- Modification of platy crystal to dendritic on application of magnetic field.
- Reduction in particle size of HAP in the presence of magnetic field.
- Magnetic field orients the growth of DCPD crystals.
- Magnetic field makes the samples devoid of HPO₄²⁻ phase.



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ABSTRACT

Iron doped calcium phosphate having different morphologies has been synthesized using gel medium and the change in morphology caused due to the incorporation of iron in the presence and absence of magnetic field have been studied. The oriented dendritic growth of brushite crystals were observed in the presence of magnetic field. The plate like hydroxyapatite (HAp) was converted into needle like morphology on application of magnetic field. Various functional groups corresponding to HAp and dicalcium phosphate dihydrate (DCPD) were observed in the FTIR and Raman spectrum. Presence of HPO₄²⁻ in the crystals grown in the absence of magnetic field has been confirmed from FTIR and Raman results. Nanoroughness of the crystals was also seen to increase on applying a magnetic field of the order of 0.1 T. Magnetic field makes the samples devoid of HPO₄²⁻ phase and also limits the concentration of iron incorporated thus, leading to a change in the morphology.

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1. Introduction

Calcium phosphates were found to exist with varying Ca/P molar ratio ranging from 0.5 to 2.0. Hydroxyapatite is a phase of calcium phosphate with Ca/P of 1.67. It is a biocompatible and bioactive material which is the main inorganic component of bone and teeth. Hydroxyapatite $[Ca_{10}(PO_4)_6 (OH)_2, (HAp)]$ has been widely employed as a bone filler. Apart from bone filler application, HAp has also been employed as coating [1], polymer composites as a drug delivery agent and as a biomarker [2,3]. Dicalcium phosphate dihydrate (DCPD), Tricalcium phosphate (TCP) and Tetra-calcium phosphate which are other phases of calcium phosphate have been used as bone cements [4,5].

Studies on the incorporation of metal ions into HAp lattice leading to a modification in its physicochemical and biological properties have been done previously by many researchers. Among these reports, HAp doped with 0.1 M concentration of iron showing rod like structure with less than 2% hemolysis has been the motivation for the present work [6]. Similarly, high hemocompatibility and antimicrobial activity were observed on Iron and zinc ions co-doped HAp [7] and Copper and zinc doped hydroxyapatite has also been used as an antimicrobial agent [8].

HAp with specified morphologies is seen to be better suited for various biomedical applications when compared to the others. They also exhibit different photoluminescent properties. It has been reported that rod like morphology of HAp adsorbs relatively more proteins than spherical and fibroid like morphologies [9] and the biocompatibility of HAp also depends on the protein adsorption and desorption on the material's surface [10]. The difference in morphology of HAp also influences the intensity of photoluminescence [11].

Various synthesis techniques have been employed to prepare HAp. Rod like morphology with controlled size has been synthesized by surfactant assisted hydrothermal method [12]. Whisker and flower like morphology of HAp have been grown in gel medium. The effect of external magnetic field on the morphology of the crystals during synthesis has been studied and it has been concluded that Fe₃O₄ nanoparticles with square like morphology were modified to wire on application of a magnetic field (0.15 T) [13]. Another report on the enhancement of the size of the brushite crystals in gel medium while application of a magnetic field of 0.1 T is available in the literature [14]. The growth of calcium phosphate crystals were extensively studied in gel medium [15,16]. To the best of our knowledge reports on the influence of magnetic field on the incorporation of iron, paving way to the growth of calcium phosphate crystals with unique morphologies are unavailable. Thus, in this study, the influence of iron and magnetic field on the growth of calcium phosphate crystals in gel medium is examined at ambient temperature and neutral pH and their physicochemical analysis are carried out.

2. Materials and method

2.1. Experimental procedure

2.1.1. Materials

Calcium nitrate tetrahydrate (Ca(NO₃)₂ 4H₂O, Merck), diammonium hydrogen phosphate ((NH₄)₂HPO₄, Fisher Scientific), ferric chloride (FeCl₃, Fisher Scientific), (Na₂SiO₃ 9H₂O, Fisher Scientific) all having > 98% purity were used for this experiment. All experiments were performed using triple distilled water.

2.1.2. Methods

Silica gel was prepared by mixing sodium meta silicate (SMS) solution of specific gravity 1.03 g/cc with 0.6 M of diammonium hydrogen phosphate in 1:1 ratio. The pH of the solution was adjusted to 7 by treating with 10% acetic acid. 10 ml of the above solution was allowed to set in the test tubes for two days.

Ferric chloride (0.1 M) was dissolved in a solution containing calcium nitrate tetra hydrate (1 M). The prepared solution was added as



Fig. 1. Schematic diagram of the formation of Liesegang rings, DCPD and spherulites formed in the gel medium (a) without magnetic field (b) with magnetic field.

supernatant and allowed to diffuse into the gel medium. The same experiment was also carried out in the presence of magnetic field by placing the test tubes in between $(Nd_2Fe_{14}B)$ bar magnets placed approximately 1 cm apart. The field strength measured using gauss meter was found to be of the order of 0.1 T. A schematic diagram of the experimental procedure is shown in Fig. 1. A photograph of the gel medium during the crystallization process is shown in Fig. 2. The Liesegang rings (LRs), DCPD and the spherulites crystallize in the gel medium due to variation in the temperature, pH, concentration, etc.



Fig. 2. Photograph of Liesegang rings, DCPD and spherulites crystallized in the gel medium (a) without magnetic field (b) with magnetic field.

during the diffusion process [14–16]. The LRs, spherulites and crystals were harvested after 15 days.

2.1.3. Observation

After the addition of supernatant solution, formation of Liesegang rings was observed within 1 h followed by the formation of dicalcium phosphate dihydrate (DCPD) in 2 days. Along with these, spherulites were also observed at the bottom of the test tubes. Iron doped Liesegang rings formed near the interface in the absence of magnetic field are labelled as FH and iron doped Liesegang rings in the presence of magnetic field are labeled as FHMF. For easy understanding, iron doped DCPD formed in the absence of magnetic field will be abbreviated as FD and iron doped DCPD in the presence of magnetic field will be mentioned as FDMF throughout. similarly, iron doped spherulites formed without magnetic field will be presented as FS and iron doped spherulites in the presence of magnetic field will be represented as FSMF henceforth.

2.2. Characterization

The crystals obtained in the gel matrix were observed under Zeiss Optical Microscope. The XRD analysis was carried out using X-ray diffractometer (XRD, Ultima IV, Rigaku, Japan). FTIR spectra was recorded with a Jasco Fourier transform infrared spectrometer (FTIR-6300) KBr pellet technique for the range of $4000-400 \text{ cm}^{-1}$ in transmission mode and Raman spectra was observed using Labram-HR 800 spectrometer equipped with an argon ion laser and the excitation was done by laser radiation of wavelength 488 nm. The SEM micrographs and EDX analysis are carried out by Carl Zeiss MA 15/EVO 18 microscope. The Atomic absorption studies were done by Atomic Absorption Spectrometer Thermo iCE 3300. The surface topography of the crystals was studied using Park XE-100 Atomic Force Microscope (AFM).

3. Results and discussion

3.1. Powder X-Ray diffraction (XRD) analysis

The XRD patterns of the FH and FHMF samples matched well with the pattern obtained for hydroxyapatite (HAp) (JCPDS (09-0432)) (Fig. 3 a–b). In the presence of magnetic field, no phase change is observed on incorporation of iron into the gel matrix. Only a slight variation in the lattice parameters is observed, when compared to the control.

The XRD patterns of the crystals grown inside the gel medium (FD and FDMF) are found to be in agreement with the pattern for dicalcium phosphate dihydrate with the JCPDS No. (09-0077) (Fig. 3 c–d). In the presence of magnetic field, the intensity of major plane (020) decreased and the intensity of (170) plane increased, indicating the oriented growth of crystals. Fig. 3(c–d), shows the patterns obtained for DCPD crystals without and with magnetic field. In the absence of magnetic field (Fig. 3c), the crystals are platy. The crystals are seen to grow only in some directions while the growth of other planes are suppressed, whereas in the presence of magnetic field (Fig. 3 d), stimulated growth of DCPD in all directions is observed. This occurs due to a phase transformation until reaching a stable phase. The mechanism of phase transformation during mineralization has already been reported [17,18].

The lattice parameter of HAp (FH & FHMF) and DCPD (FD & FDMF) crystals were calculated using XRDA software and are tabulated in Table 1. There is not much variation in the lattice parameter of FH and FHMF. However, FDMF shows a significant decrease (48%) in the c-axis compared to that of FD.

3.2. FTIR spectroscopy

FTIR spectra of the Liesegang rings (LR) and the crystals are shown



Fig. 3. XRD patterns of the samples (a) FH, (b) FHMF, (c) FD and (d) FDMF.

Table 1	
Lattice parameters	of grown crystals.

Synod	Sample codes	a (Å) ± 0.001	b (Å) ± 0.001	c (Å) ± 0.001
1	FH	9.199	9.199	6.801
2	FHMF	9.228	9.228	6.829
3	FD	6.498	15.004	8.600
4	FDMF	6.387	15.144	5.842

in Fig. 4. The LRs (FH and FHMF) showed the presence of functional groups corresponding to HAp (Fig. 4 (a) and Figure (b)). The broad band observed at 3402 cm⁻¹ corresponds to the OH stretching vibration of water. The absorption peak observed at 2352 cm^{-1} is attributed to atmospheric CO_2 . The band at 1645 cm^{-1} corresponds to the deformation vibration of OH⁻ group of water. The band at 1384 cm⁻¹ is due to the N-O stretching from the unreacted calcium nitrate adsorbed on the surface. The peaks at 1031 cm^{-1} and 1095 cm^{-1} are assigned to the asymmetric stretching mode of PO₄³⁻ group. The bands at 875 cm⁻¹ and 913 cm^{-1} indicate the bending mode of CO_3^{2-} group [19]. The bands at 560 cm^{-1} and 600 cm^{-1} correspond to the bending vibrations of P-O mode whereas, the peak 965 cm^{-1} is due to the symmetric P-O stretching vibration. On the application of magnetic field, no significant variations were observed in the functional groups of HAp. FTIR spectra of FD and FDMF are shown in Fig. 4 (c) and Fig. 4 (d) respectively. The bands observed around 3100-3600 cm⁻¹ correspond to the vibrations of the water molecule. The peak at 2357 cm^{-1} is attributed to the absorption of atmospheric carbon dioxide. The band observed at 1652 cm^{-1} is due to the presence of water. The peaks at 1221 cm^{-1} and 1136 cm⁻¹ are due to the P=O stretching vibrations of phosphate groups [20]. The vibrational mode observed at 1064 $\rm cm^{-1}$ corresponds to the asymmetric stretching mode whereas, the bands at 797 cm^{-1} , 874 cm^{-1} and 992 cm^{-1} are attributed to the symmetric stretching modes of the PO_4^{3-} group. The peaks at 664 cm⁻¹, 572 cm⁻¹, 527 cm⁻¹



Fig. 4. FT-IR spectra of (a) FH, (b) FHMF, (c) FD and (d) FDMF.

and 534 cm^{-1} are ascribed to the (HO)P = O of acid phosphates [21]. In the presence of magnetic field, the bands of DCPD crystals are significantly suppressed due to the structural change. The obtained structural changes correlate well to the structural changes seen in the XRD pattern thus, supporting the same.

3.3. Raman spectroscopic analysis

The Raman spectra for the crystals grown in the absence of magnetic field and in its presence are shown in Fig. 5 a and b respectively. Both the spectra show the presence of bands in the range of 400 cm^{-1} to 500 cm^{-1} corresponding to the doubly degenerated symmetric



Fig. 5. Raman spectra of (a) FD and (b) FDMF crystals.

Tabl	e 2		
Iron	concentration	grown	crystals

S.No.	Sample code	Iron concentration (± 2 ppm)
1	FH	106
2	FHMF	77
3	FD	33
4	FDMF	16

bending of O-P-O of DCPD, bands in the range of $510 \,\mathrm{cm}^{-1}$ to $590 \,\mathrm{cm}^{-1}$ which are attributed to the triply degenerated asymmetric bending of O-P-O and peaks for the stretching of P-O of DCPD at $988 \,\mathrm{cm}^{-1}$ and at $1122 \,\mathrm{cm}^{-1}$. In the absence of magnetic field, two other bands at $874 \,\mathrm{cm}^{-1}$ and $1055 \,\mathrm{cm}^{-1}$ corresponding to HPO_4^{2} are observed, indicating its presence in the crystal lattice [22]. In calcium phosphate, the peaks at 949 and 970 cm^{-1} were associated to internal vibrations of the PO_4^{3} ions whereas, the bands (150-300 cm^{-1}) correspond to lattice modes [23,24]. Jingwei Xu et al. reported that the FT-Raman spectra and the pressure dependence of the infrared spectra of the hydrated and anhydrous forms of dicalcium phosphate revealed a phase transition at 21 kbar and no transition respectively observed [25].

3.4. Atomic absorption spectroscopy

The iron concentration in the samples is quantified and tabulated in Table 2. Incorporation of iron was high in the absence of magnetic field when compared to the case wherein the crystals were grown in the presence of magnetic field. This result shows that the increase in the concentration of incorporated iron has led to an increase of the lattice parameters, which may be attributed to the partial replacement of calcium ions by Fe ions as reported earlier by Haishan Shi et al. [22]. Magnetic field limits the incorporation of iron on the crystals. The iron concentration in HAp was high compared to that of DCPD and temperature can also influence the incorporation of iron on DCPD crystals [16].

3.5. Optical microscopy

The optical microscope images of FD and FDMF crystals are shown in Fig. 6 a & b respectively. The FD crystals are seen to grow only in a plane and they are platy whereas, FDMF crystals grow in all the directions and have a dendrite like structure. The dendritic structures are opaque in nature which could be due to the high supersaturation of reactants. The formation of dendrites is the next stage of the platy crystals whereas in the presence of magnetic field, the initial formation of the crystal itself is in dendritic form [26].

3.6. Scanning electron microscopy

A difference in morphology was observed in the case of crystals grown under the influence of magnetic field (FHMF) when compared to those grown in the absence of magnetic field (FH). The FH and FHMF samples show plate-like morphology and needle like morphology



Fig. 6. Optical Microscope images of (a) FD and (b) FDMF.



Fig. 7. SEM micrographs of the LS, Spherulites and crystals obtained from the gel medium (a) lower magnification of FH, (b) higher magnification of FH, (c) lower magnification of FHMF, (d) higher magnification of FHMF, (e) lower magnification of FS, (f) higher magnification of FS, (g) lower magnification of FSMF, (h) higher magnification of FSMF, (i) triangular etch pits observed on the surface of FD and (j) triangular etch pits observed on the surface of FDMF.

respectively (Fig. 7(a–d)). The FS samples show rod-like morphology (Fig. 7(e–f)) whereas, the FSMF samples show elongated needle-like morphology radiating from the centre (Fig. 7(g–h)). In the presence of magnetic field, the size of the crystals (FDMF) increased when compared to those grown in the absence of magnetic field (FD). This modification in the structure and size of the crystal suggests the



Fig. 8. AFM images of FD and FDMF.

prominent role of magnetic field in the nucleation and growth of HAp. Meenakshi Sundaram et al. has reported a similar observation and explained that this phenomenon is due to an increase in the free energy of the nuclei [14,27]. Triangular etch pits are observed in the FD and FDMF crystals. Giocondi et al. demonstrated that the brushite grows in the form of triangular hillocks which eventually indicate the initiation of dissolution. The brusite crystal having triangular etch pits and the hillocks were grown on the (010) faces [28]. Similarly, the FD crystals showed bigger oblated triangular etch pits when compared to FDMF (Fig. 7 (i)).

3.7. Atomic force microscopy

The AFM images of FD and FDMF crystals are shown in Fig. 8 a & b respectively. The roughness of the FD crystals is found to be 35 nm and that of FDMF is observed to be 512 nm. An increase in surface roughness of the crystal in the presence of magnetic field is also observed in the SEM analysis, which could be due to the enhancement in the diffusion process. The rate of dissolution of the crystal increases the formation of pits leading to an increase in the surface roughness [29]. Also it has been reported, that ionic bonding between the Ca²⁺, HPO₄²⁻ ions and the growing faces of the crystal also affects the roughness of DCPD. The increase in the growth rate of the crystals affects the growing faces of the DCPD which eventually leading to an increase in the surface roughness [30]. Similar observations were found in the present study. The FD crystals are orderly arranged in an array as depicted in Fig. 8 (a) which evidences the increase in lattice parameters along the c-axis.

4. Conclusion

Platy, dendritic and spherulitic calcium phosphates were crystallized in the presence of magnetic field. The effect of incorporation of iron into the samples in the presence as well as in the absence of magnetic field have been investigated. The growth of DCPD crystals has been confirmed using XRD, FTIR and Raman studies. The presence of magnetic field lowers the incorporation of iron, leading to the formation of crystals in different planes, with a dendrite like morphology is observed. Whereas, in the absence of magnetic field platy crystals containing higher iron concentration, exhibiting additional Raman peaks corresponding to HPO_4^{2-} are obtained. The presence of magnetic field led to the growth of crystals which contain only PO_4^{3-} (i.e.) the crystals are devoid of HPO_4^{2-} phase. The change in morphology to dendrites and needles on application of magnetic field makes them suitable candidates for protein adsorption and studies on biomineralization of calcium phosphates.

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Chemical and structural analysis of gallstones from the Indian subcontinent



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ABSTRACT

Representative gallstones from north and southern parts of India were analyzed by a combination of physicochemical methods: X-ray diffraction (XRD), infrared spectroscopy (IR), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), CHNS analysis, thermal analysis and Nuclear Magnetic Resonance (NMR) spectroscopy (¹H and ¹³C). The stones from north Indian were predominantly consisting of cholesterol monohydrate and anhydrous cholesterol which was confirmed by XRD analysis. FTIR spectroscopy confirmed the presence of cholesterol and calcium bilirubinate in the south Indian gallstones. EDX spectroscopy revealed the presence of carbon, nitrogen, oxygen, calcium, sulfur, sodium and magnesium and chloride in both south Indian and north Indian gallstones. FTIR and NMR spectroscopy confirmed the occurrence of cholesterol in north Indian gallstones. The respective colour of the north Indian and south Indian gallstones was yellowish and black. The morphology of the constituent crystals of the north Indian and south Indian gallstones were platy and globular respectively. The appreciable variation in colour, morphology and composition of south and north Indian gallstones may be due to different food habit and habitat.

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1. Introduction

Cholelithiasis is not an uncommon problem encountered in day to day medical practice. Gallstones vary from patient to patient in number, size, type, shape, and chemical composition. The determination of various elements in gallstones becomes important not only in fundamental research but also to improve the patient care.

Gallstones are either cholesterol, pigment or of mixed type. The composition and characteristics of gallstones vary in different parts of the world, with cholesterol being dominant in the western hemisphere and pigment/mixed type being dominant in South Asian countries, with a shift to cholesterol type in these countries. Wide variations in the composition of gallstones have also been reported for the Indian subcontinent [1–4]. Cholesterol gallstones are predominant in the northern, eastern and western parts of India, whereas pigment gallstones are common in South India [5]. The mechanism responsible for these wide variations within a country is not clear. Several theories have been

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proposed to explain the mechanism of the formation of various types of gallstones [6].

One proposition has been the altered composition of bile. The primary lipid components of bile are bile acids, phospholipids, and cholesterol. Other major constituents of bile include water, lipids, electrolytes (Na and K), fatty acids, polysaccharides, carbonate and proteins [7]. Lithogenic bile is likely to predispose an individual to cholesterol gallstones [8]. The process of crystal nucleation in cholesterol gallstones is normally slow within the gallbladder. Supersaturated bile remains metastable for many days. During gallstone formation, this metastability is lost and crystal formation occurs within hours. Further, growth of gallstone occurs either by deposition of additional insoluble precipitants at the bile/stone interface or via a process of agglomeration of cholesterol crystals [9].

Little is known about the pathogenesis of pigment gallstones. They are probably the result of supersaturation of bile with calcium salts of bilirubin, phosphate, carbonate and other anions. Hemolytic anemia is an established cause of the formation of pigment gallstone, but in its absence, the pathogenesis of these stones is less clear [10]. Bacterial infection within the biliary system also predisposes a person to pigment or mixed gallstones [11]; mechanisms are unclear. It has been reported



Fig. 1. Optical micrographs of the gallstones: (a and b): South Indian gallstones (G1 and G2) and (c, d and e): North Indian gallstones (G3, G4 and G5).

that *Tamarindus indica*, an integral component of south Indian cuisine may be an important risk factor for pigment gallstone formation [12–13]. It has been also shown earlier the high iron content in pigment gallstones and tamarind (*Garcinia camborginia*) by PIXE analysis [14] and hypothesized that possibly iron in ferrous form served as nucleation site for pigment gallstone formation.

Mixed gallstones are composed of both cholesterol and calcium bilirubinate. Possibly, the same pathogenic mechanism of cholesterol and pigment stones may be involved in the formation of these gallstones [15–16]. Apart from some of these known factors, geological location, other components in bile such as sex hormones, glycoproteins, fatty acids, a person's nutrition habits, and finally liver-associated diseases may be responsible for the nucleation and the formation of these types of gallstones [17]. As the pathogenesis of gallstones still remains elusive we undertook an in-depth study of the physicochemical analysis (XRD and IR), SEM, EDX analysis, thermal analysis, elemental analysis, and ¹H and ¹³C NMR spectroscopy of gallstones (pigment, mixed and cholesterol) were carried out.

2. Materials and methods

2.1. Epidemiological studies

Gallstones were collected from 20 patients undergoing cholecystectomy for symptomatic gallstone disease at Jaswant Rai Specialty Hospital, Meerut, Uttar Pradesh, to represent the northern part of India, and 37 samples from Global Health City, Chennai, Tamil Nadu, to represent South India. The age of the patients was between 28 and 58 years.

The stones were rinsed several times with deionized water and dried at room temperature. The samples were labeled as mixed gallstones, pigment gallstones and cholesterol gallstones. Representative gallstone



Fig. 2. XRD patterns of the gallstones.



Fig. 3. IR spectra of gallstones samples (a) G1, (b) G2, (c) G3, (d) G4 and (e) G5.

samples 2 each from Tami Nadu and 3 from Uttar Pradesh were analyzed for structural and chemical characteristics.

2.2. General observation of gallstones

The selected gallstones were photographed and examined under an optical stereo microscope for their colour and surface morphology (Fig. 1a–e). The south Indian gallstones were coded as G1 and G2. G1 (Fig. 1a) was dark brown with a well-defined rough outer surface. The G2 stone (Fig. 1b) was black, irregular in shape and was harder than the other gallstones. The north Indian gallstones were coded as G3, G4 and G5. The G3 was yellowish-brown with a very smooth surface whereas, the G4 was greenish-yellow with a rough outer surface. G5 was pale yellow, faceted with a well-defined smooth surface.

2.3. Characterization

All the samples were ground to have a homogeneous mixture for analysis. The phase identification of the gallstones was carried out by XRD using Bruker D8 Advance instrument with CuK_{α} radiation ($\lambda = 1.5406$ Å; 40 kV; 40 mA; 5° to 90° 20; step size 0.01° 20 s⁻¹; room temperature).

Table 1

Vibrational assignments of cholesterol (G1, G3, G4, G5).

Assignments	Vibrational frequency (cm^{-1})
CH asymmetric stretching mode of CH ₂	3380, 3396
CH asymmetric stretching mode of CH ₃	2919, 2936, 2982
CH symmetric stretching mode of CH ₃	2896, 2849, 2898
CH symmetric stretching mode of CH ₂	2849, 2856, 2863, 2864
CH bending mode of CH ₂	1457, 1458, 1461
CH bending mode of CH ₃	1367, 1373
C-C stretching mode	1050, 1051
C-C-C bending mode	493

Table 2

Vibrational assignments of carbonates and phosphates.

Assignments	Vibrational frequency (cm ⁻¹)
C-O stretching of CaCO ₃	1444
C-C stretching	1175
Stretching mode of PO ₄ ³⁻	962
C-O bending of CaCO ₃	837
Bending mode of PO ₄ ³⁻	563,605

Table 3

Vibrational assignments of bilirubin.

Assignments	Vibrational frequency (cm ⁻¹)
OC==O stretching mode	1647
C==C stretching mode	1614, 1569
CO stretching mode in HOC==N grouping	1244
C—H in plane bending mode	1175
CCH plane bending mode	1044
CC ring stretching mode	982



Fig. 4. SEM micrographs of gallstones (a) lower magnification of G1, (b) higher magnification of G1, (c) lower magnification of G2, (d) higher magnification of G2, (e) lower magnification of G3, (f) higher magnification of G3, (g) lower magnification of G4, (h) higher magnification of G4, (i) lower magnification of G5 and (j) higher magnification of G5.

Table 4					
Elements prese	ent in the gallstones	G1, G2,	G3, G4	and G5	5.

Sample	Carbon (wt%)	Nitrogen (wt%)	Oxygen (wt%)	Calcium (wt%)	Sulfur (wt%)	Sodium (wt%)	Magnesium (wt%)	Chlorine (wt%)	Silicon (wt%)
G1	88.4 ± 0.5	-	11.4 ± 0.5	Traces	-	-	-	-	-
G2	62.1 ± 0.8	5.2 ± 0.6	26.9 ± 1.4	2.1 ± 0.4	2.3 ± 0.8	Traces	Traces	Traces	-
G3	87.9 ± 1.4	-	10.5 ± 0.6	Traces	-	Traces	Traces	Traces	Traces
G4	89.1 ± 1.0	-	10.5 ± 0.9	Traces	-	Traces	-	Traces	-
G5	87.7 ± 1.8	Traces	11.5 ± 0.5	Traces	Traces	Traces	-	Traces	-

The functional groups of the samples were identified through the FTIR analysis. The IR spectra of the samples were recorded by a Jasco Fourier transform infrared spectrometer MODEL IR-6300 in KBr pellets. The spectra were recorded from 400 to 4000 cm⁻¹ in transmission mode.

The NMR spectroscopy is an extensive tool to study the chemical structure of the samples. The analysis was carried out with a Bruker DPX 300 instrument (300 MHz). The ground samples were extracted with CDCl₃ at room temperature and the insoluble residue was filtered off. CDCl₃ served as internal standard (¹H: δ = 7.24 ppm; ¹³C: δ = 77 ppm). The SEM is employed to study the surface morphology of the samples. The SEM was carried out with an ESEM Quanta 400 FEG instrument (FEI) on Au/Pd (80/20) sputtered samples. The EDX was used to study the elemental analysis of the samples. The analysis was carried out with an EDAX-EDS analysis system type Genesis 4000 at the same electron microscope. The rapid determination of carbon, hydrogen, nitrogen and sulfur in organic matrices of the samples was carried out by CHNS (combustion analysis) analysis using a Euro Vector EA 3000 analyzer.

3. Results and discussion

3.1. XRD analysis

The XRD patterns of the gallstones are shown in Fig. 2. The patterns of G1, G3, G4 and G5 were in good agreement with the standard patterns of cholesterol which correspond to the triclinic space group P1. Konikoff et al. and Kumar et al. have studied the crystallization in organic solvents and XRD analyses, confirmed the formation of anhydrous and monohydrate cholesterol [18-22]. The XRD pattern of G1 revealed the presence cholesterol monohydrate as reported in the literature [23]. Sutor and Wooley had reported that the pigment stones were organic and amorphous. Similarly, the G2 stone did not exhibit any diffraction peaks which confirmed its amorphous nature [24]. The XRD patterns of the mixed gallstones G3 and G4 indicated the presence of both anhydrous and monohydrated cholesterol, with the prominent peak of cholesterol monohydrate at 5.3° along with other characteristic peaks at 14.7° and 14.9°. The peaks corresponding to anhydrous cholesterol were observed at 12.9° and 14.0°. XRD pattern of G5 was identical to that of anhydrous cholesterol. Cholesterol gallstones were formed in two steps. In the first step, the bile probably forms precipitates containing microcrystals of cholesterol. In the second step, the microcrystals of cholesterol subsequently grow into macroscopic gallstones. The presence of insoluble cholesterol lead to the enhancement of cholesterol levels in the bile. Generally, the gallstones were formed due to the supersaturation of unconjugated bilirubin and other bile salts [25]. The

Table 5				
Elemental analysis of gallstones G1.	, G2,	G3	G4 and	G5.

Sample	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulfur (%)	Calcium by AAS (%)
G1 G2	77.8 ± 0.1 50.5 ± 0.2	11.1 ± 0.2 63 ± 0.2	0.76 ± 0.02 8 90 + 0.09	-1.48 + 0.04	0.31
G3	-	-	-	-	0.86
G4	80.0 ± 0.2	11.6 ± 0.1	0.10 ± 0.01	-	0.22
G5	75.6 ± 0.6	10.3 ± 0.4	0.63 ± 0.04	-	0.54

collected gallstones were identified by XRD analysis as mixed gallstones, pigment gallstones and cholesterol gallstones (Fig. 2).

3.2. FTIR analysis

The collected gallstones were analyzed by FTIR to identify the presence of functional groups (Fig. 3). The FTIR analysis revealed the presence of calcium bilirubinate in the pigment stone (G2) and moreover; carbonate and phosphate occurred in all the samples. FTIR spectroscopy provided deeper comprehension of individual molecules in gallstones. G1, G3, G4 and G5 showed the characteristic peaks of cholesterol. Suzuki and Toyoda reported that the infrared absorption spectra of calcium bilirubinate and bilirubin of the gallstone. G2, the pigment stone exhibited the prominent characteristic peaks of calcium bilirubinate (Table 3) [26]. The presence of the bilirubin in the black pigment stone (G2) was confirmed by the peak corresponding to C—O stretching mode of HO—C=N group of bilirubin at 1244 cm⁻¹. Kleiner et al. reported the varying amount of bilirubin in the black pigment stones [27]. The carbonate and phosphate groups were identified in all the samples. Kothai et al. have also reported the presence of carbonate and phosphates in the gallstone [28]. The bands due to the bending mode of the phosphate groups were observed at 563 cm^{-1} and 605 cm^{-1} whereas, the band at 962 cm^{-1} and at 1444 cm⁻¹ were due to the stretching mode of the phosphate and C—O stretching of carbonate groups respectively. The major chemical components of gallstones and their corresponding IR bands are provided in Tables 1 to 3 [29].



Fig. 5. TGA/DTA analysis of representative (a) G2 and (b) G4 samples.



Fig. 6. Carbon atom positions in the Structure of cholesterol.

3.3. SEM

The SEM micrographs of the surface of the gallstones are shown in Fig. 4a-j. Garti et al. reported that crystals of plate-like and needle-like morphology corresponds to the cholesterol monohydrate and cholesterol anhydrate respectively. Similar morphology was observed in the G1, G3, G4 and G5 samples [20]. Whereas, Bills et al. reported that cholesterol stones possess a layered structure which was built up in step-by-step fashion around the small center [30]. The samples G1 (Fig. 4a-b), G3 (Fig. 4e-f) and G4 (Fig. 4g-h) contained platy and needle shaped crystals. In G1, the length of needles is 8 \pm 2 µm. The plate-like crystals were found to be large in quantity compared to the needle type crystals and their length and breadth were $3 \pm 1 \,\mu\text{m}$ and $0.8 \pm 0.1 \,\mu\text{m}$, respectively. The G2 exhibited both globular and irregular flake like morphology formed due to the lateral growth. The G3 consisted of platy crystals having an average length of 4 ± 0.8 µm and breadth 1 ± 0.4 µm along with the needle like crystals (5 \pm 1 μ m). The G4 consisted of platy crystals having length and breadth of 6.0 μ m \pm 0.2 μ m and 2.0 μ m \pm 0.1 μ m respectively. The platy crystals were clustered together to form the bulk structure. The needles were found in very small quantity having a length of 3 \pm 0.7 μ m. The G5 (Fig. 4i–j) consisted of flake like crystals $(15 \pm 5 \,\mu\text{m} \times 5 \pm 4 \,\mu\text{m})$. The reason for the formation of different morphologies of gallstones is still not clear.



Fig. 8. Hydrogen atom positions in the Structure of cholesterol.

3.4. EDX analysis

The EDX elemental concentrations are given in Table 4. Cavalu et al. reported the black and brown pigment stones have the coordination of Cu (II) with four nitrogen atoms forming the tetrapyrrole rings of bilirubin. Carbon, oxygen and trace amount of calcium were detected in all types of gallstones by EDX analysis. In comparison with the Cavalu et al., the higher nitrogen content was found in G2 stone [31]. The low carbon content in G2 indicated a smaller amount of cholesterol in the pigment stones. Moreover, in G2, high level of oxygen was detected when compared to the other samples.

3.5. CHNS analysis

The content of carbon, hydrogen, nitrogen and sulfur in the samples was obtained by elemental analysis (CHNS) (Table 5). This analysis was supported by the EDX data. Usually, the core of the pigment stones



Fig. 7. ¹³C NMR spectra of (a) G1, (b) G3, (c) G4 and (d) G5.

contains sulfur and large amounts of calcium and phosphorus. Bills and Jewis reported the presence of sulfur in the organic pigment stones [30]. The presence of sulfur in the center of the black pigment stone was demonstrated from the electron probe microanalysis by Malet et al. [32]. Similarly, the sulfur content was found only in G2 pigment stone. Trotman and Soloway analyzed the composition of the black pigment stone and found calcium carbonate and phosphate other than calcium bilirubinate in a glycoprotein matrix with low cholesterol content [33]. The nitrogen content was eight times higher in G2 compared to that of other gallstones due to the degraded tetrapyrrolic bile pigments derived from bilirubinates (biliary infection occur in gallbladder and bile ducts) and bilirubin (excreted in bile and urine) [31]. Whiting et al. revealed the low cholesterol content of the black pigment stones [34]. The cholesterol was very low in pigment stones (G2), and thus the carbon content was also less [33]. In all cases, traces of Mg and Na were detected by AAS (always below 0.2 wt%). The G3 was not available in sufficient quantity for CHNS elemental analysis. Elemental analysis revealed the presence of high quantity of nitrogen, sulfur and calcium in pigment stones (G2) in comparison with other samples.

3.6. Thermal analysis

TGA/DTA analysis of G2 and G4 was shown in the Fig. 5. The thermal decomposition of gallstone samples was observed in two consecutive steps at different temperatures. The weight loss in G2 (4%) at 85 °C was due to loss of water molecule, whereas; in G4 weight loss was observed at 290 °C. The second weight loss detected in G2 and G4 samples were 31% and 47% respectively. In G2, the second weight loss began at 250 °C due to minor quantity of cholesterol present in sample therefore, there was leading to a gradual weight loss. The third weight loss of G4 at 340 °C was due to the melting of cholesterol. The G4 was found to be stable beyond 480 °C, which might be due to the presence of inorganic

constituents like calcium phosphate, calcium bilirubinate, calcium oxalate etc. The residual weight of G2 (20%) was significantly high in comparison with G4 (8%), because of thermal stability of calcium phosphate or hydroxyapatite. In Fig. 5(a) (DTA), the exothermic peak was observed at 62 °C whereas, in G4 (Fig. 5(b)), the exothermic peak was found at 115 °C. The endothermic peaks of G2 were found at 260 °C, 390 °C and 480 °C however; for G4, endothermic peaks were observed at 320 °C, 360 °C and 415 °C. The low residual weight loss in pigment stone confirms the presence of calcium phosphate and calcium bilirubinate [35–37].

3.7. ¹³C NMR and ¹H NMR

The structure of cholesterol with the carbon and hydrogen atom is labeled in Fig. 6 and Fig. 8 respectively. ¹³C and ¹H NMR spectra of the $CDCl_3$ extracts of the gallstones are as shown in the Fig. 7(a-d) and Fig. 9(a-d) respectively. Javalakshmi et al. found that the most of the gallstones have cholesterol as its main constituents using ¹³C CP-MAS NMR experiments [38]. Chemical shifts of carbon and hydrogen of cholesterol were in good agreement with the values reported in the literatures [39–40]. Cholesterol was detected in all the samples except in G2, where no soluble species were found in it (no NMR signals). Craven reported that carbons (C5, C6, C9, and C18) having two peaks with almost equal intensities in cholesterol monohydrate and existed in bilayers [39]. Here in this case, the carbons (C5, C6, C9 and C18) with different intensities were probably observed due to different biochemical environment in which it was formed. The hydrogen atom peak positions in the ¹H NMR spectrum confirmed the presence of cholesterol. Water was not detected in any of the samples by ¹H NMR spectroscopy which was also confirmed by FTIR analyses. The respective chemical shifts of H6 and H3 of cholesterol observed in NMR spectra at



Fig. 9. ¹H NMR spectra of (a) G1, (b) G3, (c) G4 and (d) G5.

Table 6

Summary of the characterization studies of the gallstone samples.

Sample	Optical microscopic observation	X-ray diffraction	Infrared spectroscopy	Scanning electron microscopy	EDX	Combustion analysis and AAS	NMR (¹ H and ¹³ C)
G1	Outer surface was brown, a well-defined rough surface	cholesterol monohydrate	С—Н, О—Н, С—О, С — О	Needles/plate-like particles	C, 0	C, H, N	Cholesterol
G2	Black, irregular shaped, harder	Amorphous	С—Н, О—Н, С—О, С==О	Globular/smooth particles	C, N, O, S, Ca	C, H, N, S, Ca	No soluble organic compounds
G3	Yellowish-brown with a smooth surface	cholesterol monohydrate/anhydrate	С—Н, О—Н, С—О, С==О	Needles/plate-like particles	C, 0	Ca	Cholesterol
G4	Yellowish-brown with a smooth surface	cholesterol monohydrate/anhydrate	С—Н, О—Н, С—О, С==О	Needles/plate-like particles	C, 0	С, Н	Cholesterol
G5	Yellowish-white with a well-defined smooth outer surface	cholesterol anhydrate	С—Н, О—Н, С—О, С==0	Plate-like particles	C, O	С, Н	Cholesterol

5.35 ppm and 3.55 ppm were in agreement with that reported by S.P. Sawan et al. [40].

4. Conclusions

Table 6 summarizes the characterization studies of gallstone samples. The XRD, FTIR and NMR spectroscopy studies revealed the presence of cholesterol monohydrate and cholesterol anhydrous in the north Indian cholesterol and mixed stones and south Indian mixed stones. The EDX spectroscopy and CHNX analyses were carried to determine the elemental composition of the samples. The pigment stone was confirmed to be amorphous and calcium bilirubinate was identified as one of the constituents by FTIR spectroscopy. The constituent crystal of south Indian pigment gallstones was globular, whereas the north Indian gallstones contained platy crystals. EDX spectroscopy revealed the presence of silicon only in north Indian gallstones. However, carbon, nitrogen, oxygen, calcium, sulfur, sodium and magnesium and chloride were present in all stones. Thermal analysis also confirmed that pigment stone (G2) contained higher residual weight (20%) compared to cholesterol G4 (8%). FTIR and NMR spectroscopy were used to identify the presence of cholesterol in north Indian gallstones. The predominant constituents of south Indian gallstones were cholesterol and calcium bilirubinate confirmed by IR spectroscopy.

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