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Research paper

Further characterization of theobroma oil–beeswax admixtures as lipid matrices for improved drug delivery systems

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Abstract

There is an increasing interest in lipid based drug delivery systems due to factors such as better characterization of lipidic excipients and formulation versatility and the choice of different drug delivery systems. It is important to know the thermal characteristics, crystal habit, texture, and appearance of a new lipid matrix when determining its suitability for use in certain pharmaceutical application. It is line with this that this research was embarked upon to characterize mixtures of beeswax and theobroma oil with a view to applying their admixtures in drug delivery systems such as solid lipid nanoparticles and nanostructured lipid carriers. Admixtures of theobroma oil and beeswax were prepared to contain 25% w/w, 50% w/w, and 75% w/w of theobroma oil. The admixtures were analyzed by differential scanning calorimetry (DSC), small angle X-ray diffraction (SAXD), wide angle X-ray diffraction (WAXD), and isothermal heat conduction microcalorimetry (IMC). The melting behavior and microstructures of the lipid admixtures were monitored by polarized light microscopy (PLM). Transmission electron microscopy (TEM) was used to study the internal structures of the lipid bases. DSC traces indicated that the higher melting peaks were roughly constant for the different admixtures, but lower melting peaks significantly increased ($p < 0.05$). The admixture containing 25% w/w of theobroma oil possessed highest crystallinity index of 95.6%. WAXD studies indicated different reflections for the different lipid matrices. However, new interferences were detected for all the lipid matrix admixtures between $2\theta = 22.0°$ and $2\theta = 25.0°$. The lipid matrices containing 50% w/w and 25% w/w of theobroma oil showed absence of the weak reflection characteristic of pure theobroma oil, while there was disappearance of the strong intensity reflection of beeswax in all the lipid matrix admixtures at all stages of the study. PLM micrographs revealed differences with regard to the thermal and optical behaviors depending on the composition of the matrix. The lipid matrix consisting of 75% w/w of theobroma oil showed a spherulite texture after 4 weeks of isothermal storage. Crystallization exotherms of lipid matrices containing 50% w/w and 25% w/w of theobroma oil showed change in modification after 30 min with the latter having a greater time-dependent crystallization. Generally, low non-integral Avrami exponents and growth rate constants were obtained for all the lipid matrices, with the admixture containing 25% w/w theobroma oil having the lowest Avrami exponent and growth rate constant. Based on the results obtained, admixtures containing 50% w/w and 75% w/w of theobroma oil could be applied in the formulation of solid lipid nanoparticles and nanostructured lipid carriers as these lipid matrices possessed crystal characteristics that favour such drug delivery systems.

Keywords: Theobroma oil; Beeswax; Crystallinity; Crystallization kinetics; Differential scanning calorimetry; X-ray diffraction; Polarized light microscopy; Isothermal heat conduction microcalorimetry; Drug delivery

1. Introduction

There is an increasing interest in lipid based drug delivery systems due to factors such as better characterization of lipidic excipients and formulation versatility and the choice of different drug delivery systems [1]. With the ability to

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combine the beneficial characteristics of component fatty acids, lipid modifications may enhance the role fats or lipids play in drug delivery applications. Structured lipid matrices are tailor made with improved physical properties compared with the individual components. Long chain fatty acids ranging from C14 to C24 are common to animals and vegetable oils. A major concern with the use of animal fats as drug delivery basis for oral or parenteral administration centres on the possibility to increase the blood serum cholesterol. Long chain saturated fatty acids are generally believed to increase serum cholesterol levels. However, stearic acid (18:0) a major constituent of animal fats is neutral with respect to cholesterol levels in the blood, partly because it has melting point that is higher than body temperature and it is readily desaturated to oleic acid in vivo [2]. Natural fats may have better in vivo tolerability than synthetic fats. For instance, cocoa butter (theobroma oil) has a better biocompatibility and a lower in vivo toxicity than the semi-synthetic lipids [3].

Depending on a variety of factors, a fat may exist in one crystalline form or it may be a mixture of several different crystal modifications. Fats are polymorphic and transform systematically through a series of successive crystalline forms without change in chemical structure [4]. The polymorphic transitions may be influenced by the addition of one or more substances (or other fats) to the lipid matrix. Consequently, certain properties necessary for improved performance as drug delivery bases may be influenced. It is important to know the thermal characteristics, crystal habit, texture, and appearance of a new lipid matrix when determining its suitability for use in certain food or pharmaceutical application.

DSC is the most widely used thermo-analytic technique for studying fats, oils, and their mixtures. It gives information about the temperatures and energy associated with their fusion and crystallization, phase behavior, polymorphic transformations, and data to estimate solid fat contents [5–7]. DSC reports the destruction of structures in recordings obtained in a permanent out-of-equilibrium state [8]. X-ray diffraction, XRD (SAXD and WAXD) is also an essential tool for elucidating properties of fats and their mixtures [9–11]. However, it complements DSC. XRD recordings provide both short and long spacings at a given temperature at which the sample is supposed to be in equilibrium. Since lipid systems are quite sensitive to their preparation history, only simultaneous recordings of SAXD, WAXD, and DSC circumvent the problem of reproducibility and guarantee identical conditions for all three measurements whatever be the thermal treatment of the sample [8]. PLM is an analytical technique used in characterization of fats to observe the microstructures of the various polymorphic forms of fats. It is also used to observe the microstructural changes in fats during melting, as the lipid passes from crystalline phase to isotropic phase. IMC is a recent and an important tool in studying the time dependent crystallization of lipid matrices. It has been applied in both pure and mixed systems [12,13]. Isothermal crystallization kinetics studies of mixtures of lipids using IMC also address the question of how the crystallinity of one component affects the crystallization behavior of the other.

In this study, mixtures of beeswax and theobroma oil were further characterized for their possible improved application in novel drug delivery systems such as solid lipid nanoparticles, nanostructured lipid carriers, and solid self-emulsifying drug delivery systems. These lipids have a good track record of biocompatibility. Traditionally, beeswax has been used to stiffen theobroma oil in suppository formulations, but this practice has not been extended to other drug delivery systems. It was thus the objective of this study to further characterize physical mixtures of these two lipids which have been used for a long time in the pharmaceutical and food industries with a view to applying them to other nano- and microparticulate drug delivery systems. Beeswax is a natural fatty product obtained from honey combs of bees (*Apis mellifera*) [14]. Theobroma oil, obtained from a plant *Theobroma cacao*, has well-documented properties [15]. Admixtures of lipids have severally been studied by different authors [16–20]. However, to our knowledge, mixtures of beeswax and theobroma oil have not been studied so far.

2. Materials and methods

2.1. Materials

White beeswax (Cera alba pellets, Ph. Eur. 2002 grade) and theobroma oil (Oleum Cacao Chips, melting point range 30–35 °C, DAB 1999 grade) were purchased from Caesar & Loretz, Hilden Germany and used without further treatment.

2.2. Preparation of lipid matrices

Binary mixtures of theobroma oil and beeswax corresponding to 25% w/w, 50% w/w, and 75% w/w of theobroma oil were prepared by fusion. In each case, the lipids were weighed with an electronic balance (Sartorius AG, Type L2200P-xD2, Göttingen, Germany) and melted together at 70 °C on a hot plate (RCT basic, IKA® Staufen, Germany) and stirred until solidification.

2.3. Characterization of the lipid matrices

2.3.1. Differential scanning calorimetry (DSC)

Thermal behaviors of all the lipid matrices were determined on a calorimeter (DSC 220C) connected to a disc station (5200H Seiko, Tokyo Japan). Approximately 5 mg of each lipid matrix was weighed into an aluminium pan and sealed hermetically, and the thermal behavior determined against an empty pan in the range of 10–80 °C at a heating rate of 5 °C min^{-1}. The temperature was held for 10 min at 80 °C and thereafter, cooled at the

rate of 5 °C min^{-1} to 10 °C. The degree of crystallinity of each lipid matrix was determined by calculating the crystallinity index (CI) from the heat of fusion using a modified equation from the literature Eq. (1) [21].

$$\mathrm{CI}(\%) = \frac{\mathrm{Enthalpy}_{\mathrm{LMad}}(\mathrm{J/g})}{\mathrm{Enthalpy}_{\mathrm{BW}}(\mathrm{J/g})} 100 f_{\mathrm{BW}}, \quad (1)$$

where $\mathrm{Enthalpy}_{\mathrm{BW}}$ is the fusion enthalpy of the pure beeswax, $\mathrm{Enthalpy}_{\mathrm{LMad}}$ is the fusion enthalpy of the lipid admixture and f_{BW} is a factor which takes into consideration the concentration of beeswax in the matrix. DSC thermograms were recorded 1, 3, and 6 weeks after lipid matrices preparation. The transition temperatures were taken as the minimum temperatures of the endothermic transitions, while transition enthalpies were obtained by integration of the endothermic peaks within the temperature of 22–70 °C, using linear baselines.

2.3.2. Polarized light microscopy (PLM)

PLM is used to observe the microstructures of lipids and to monitor their melting transitions and fusion. Micrographs of crystallizing lipid matrices were examined with a Zeiss type III photomicroscope (Model No. SIP 48560, Oberkochen, West Germany) using cross polarizers and a wavelength (λ) plate. Thermal behavior of the matrices between ambient temperature and 70 °C was studied with a FP82HT Hot Stage with FP 90 Central Processor from Mettler Toledo (Gießen, Germany) with a heating rate of 5 °C min^{-1}. PLM micrographs were taken at temperatures of transitions with a digital camera (Olympus DP12, Japan) attached to the photomicroscope. The micrographs of statically crystallized lipid matrices at 25 °C were also taken after 24 h, 1 and 4 weeks.

2.3.3. Wide angle X-ray diffraction (WAXD)

To study the crystal characteristics of the lipids and their admixtures, WAXD studies were carried out using an X-ray generator PW3040/60 X'Pert PRO (Fabr.:DY2171 PANalytical, Netherlands) connected to the tube (PW3373/00 DK147726 Cu LFF) copper anode which delivered X-ray of wavelength, $\lambda = 0.1542$ nm at a high voltage of 40 kV and an anode current of 25 mA. WAXD measurements were taken with a Goniometer (PW3050/60 MPD-System, PANalytical, Netherlands) from 3.0° to 33.0° in 0.015° steps (1 s per step). The interlayer spacings were calculated from the reflections using Bragg's equation

$$n\lambda = 2d \sin\theta, \quad (2)$$

where λ is the wavelength of the incident X-ray beam, n is a positive integer which describes the order of the interference and θ is the scattering angle. The parameter d, otherwise called the interlayer spacing, is the separation between a particular set of planes of the crystal lattice structure. WAXD diffractograms were obtained 1, 7, and 12 weeks after lipid matrices preparation.

2.3.4. Small angle X-ray diffraction (SAXD)

This technique was used to analyze the long range order of the crystalline structure of the lipid matrices. Characteristic Cu–K_α radiation with a wavelength $\lambda = 0.1542$ nm was produced by an X-ray tube (PW2213/20, Feinfokus Cu-Anode) connected to a PW1730/10 generator (PANalytical Netherlands) and a Goniometer (PW1050/25, PANalytical Netherlands) was used to detect the small angle scattering. To reduce the K_β radiation, the X-ray beam was passed through a nickel filter. Tube voltage was set to 40 kV with an anode current of 25 mA. Measurement time was set at 600 s for all the lipid matrices and SAXS reference systems. Lipid samples were put in aluminium cubes and equilibrated for 10 min at 20 °C before taking the measurements. The interlayer spacings were thereafter calculated using Bragg's equation Eq. (2). SAXD diffractograms were obtained 1, 7, and 12 weeks after lipid matrices preparation.

2.3.5. Transmission electron microscopy (TEM)

The TEM of the pure lipids and the 50% w/w combination of the lipids were determined to further study the internal structures of the matrices. In a typical experiment, the lipid samples were shock-frozen in melting nitrogen at 63 K between two flat gold holders. The frozen samples were fractured at 173 K in a BAF 400 instrument (Balzers, D-Wiesbaden Germany) and then shadowed with platinum/carbon (2 nm) at 45° and with pure carbon at 90° for replica preparation. After cleaning with a chloroform–methanol mixture (1:1), the replicas on uncoated grids were viewed using a transmission electron microscope (Leo 922, Leo D-Oberkochen Germany) at 200 kV.

2.3.6. Isothermal heat conduction microcalorimetry (IMC)

IMC was used to determine the time-dependent crystallization of the molten lipid matrices. This would enable prediction of the crystallization behavior of the lipid matrices when used in formulation. A 2277 Thermal Activity Monitor® (TAM, Thermometric AB, Jarfalla Sweden) was used for this study. Two calorimeter units were installed and run concurrently. A 300 mg quantity of each lipid matrix was weighed into 3 ml glass ampoules, melted at 70 °C and equilibrated for 30 min at 20 °C, and then the heat flow measured at the range of −3000 to 3000 μW. Heat flow signals were monitored by the Digitam Software (Thermometric AB, Jarfalla Sweden). Data obtained from the IMC measurements were thereafter analyzed to determine the isothermal crystallization kinetics of the lipid matrices using the kinetics described by the Avrami equation [22,23]

$$x_t = 1 - e^{-kt^n}, \quad (3)$$

where x_t represents the crystallized fraction at time t, k (time^{-1}) is the rate constant of isothermal crystallization and n is the Avrami exponent related to the type of nucleation and growth mechanisms of crystals particularly with regards to the dimensionality of growth [22].

3. Results and discussion

3.1. Differential scanning calorimetry (DSC) measurements

A representative DSC thermogram of the lipid matrices is presented in Fig. 1 (obtained after 6 weeks), while peak endotherm and enthalpy changes are presented in Figs. 2A and B. Transition temperatures were determined from the peak minimum while enthalpy was determined by integration of the endotherms using linear baselines within the range of 22–70 °C. The result obtained for pure theobroma oil and beeswax is in consonance with other reported works [14,24]. From Fig. 1, the endothermic transitions showed bimodal endothermic peaks with the lower melting peaks due to pure theobroma oil and the higher melting peaks due to beeswax present, especially in the admixtures with 50% w/w and 75% w/w of theobroma oil. This is common with lipid mixtures [17,20]. However, there was no complete separation of the endothermic transitions pointing to the fact that there were some influences of lipids on each other. Particularly noticeable was the fact that the endothermic peak heights became smaller compared with that of theobroma oil alone. This may be attributed to the heterogeneous composition of the matrices and suggests presence of disorder in the matrices which is necessary for increased drug loading. Disorder creates spaces where drug molecules could be entrapped. For clear presentation, the variation of the enthalpies and melting peaks were analyzed graphically and presented in Figs. 2A and B. On analysis after 6 weeks, the endothermic patterns had significant changes ($p < 0.05$) in the enthalpies of the 50% w/w and 75% w/w theobroma oil-containing admixtures (Fig. 2A) and the lower melting peaks of all the admixtures (Fig. 2B). There was an insignificant increase in the melting

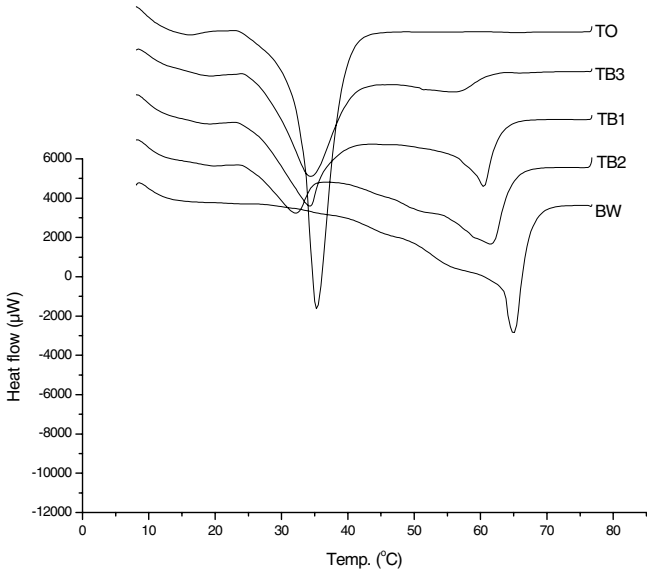

Fig. 1. DSC thermograms of the lipid matrices after 6 weeks: TO (theobroma oil), BW (beeswax), TB3 (75% w/w theobroma oil), TB1 (50% w/w theobroma oil), and TB2 (25% w/w theobroma oil).

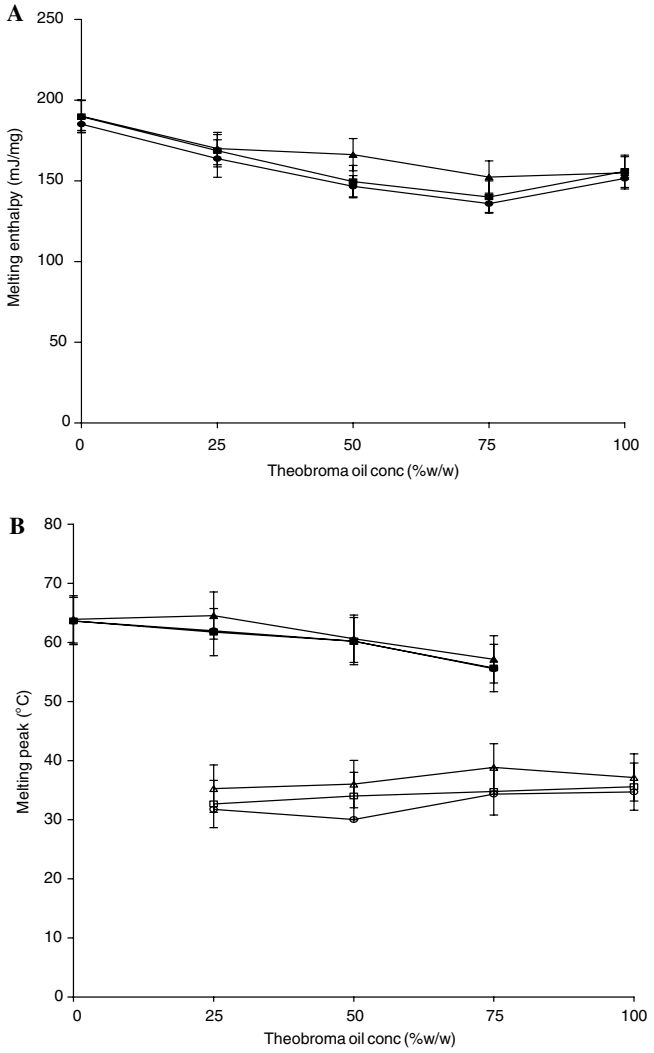

Fig. 2. Thermal properties of the lipid matrices ($n = 3$, mean ± Standard deviation): (A) melting enthalpy (● 1 week, ■ 3 weeks, ▲ 6 weeks) (B) melting peak (● highest after 1 week, ○ lowest after 1 week, ■ highest after 3 weeks, □ lowest after 3 weeks, ▲ highest after 6 weeks, Δ lowest after 6 weeks).

peak of beeswax alone from 63.6 ± 0.3 °C after 1 week to 63.9 ± 0.3 °C after 6 weeks (which is about its melting point), with minor change in enthalpy. This is expected since waxes show little or no polymorphic transition. In all the two component lipid matrices, there was significant increase ($p < 0.05$) in the lower melting peak between 1 and 6 weeks, which may be due to the transformation of the unstable α modification to more stable β modification. Analysis of the crystallinity indices (CI) revealed the following values: 95.6 ± 3.8%, 59.5.0 ± 7.3%, and 19.3 ± 5.5% for 25% w/w, 50% w/w, and 75% w/w theobroma oil-containing admixtures respectively, which were statistically significantly different from each other ($p < 0.05$). Deviations from 100% are related to the theobroma oil content. Higher theobroma oil produced lower matrix crystallinity. One should thus have in mind that greater quantity of theobroma oil would be required for products where

less matrix crystallinity is required. The overall implication of the DSC result is that these lipid matrices may form at least partially amorphous systems after melting and solidification. Crystallinity indices indicated that the admixture containing 25% w/w theobroma oil was more crystalline than all the binary lipid matrices, suggesting a higher degree of crystalline order in this lipid matrix. It is thus not a good admixture for solid lipid nanoparticles or nanostructured lipid carriers since high crystallinity would lead to drug expulsion after melting and recrystallization. For the admixture containing 75% w/w theobroma oil, the highest melting peak was lower than others after 6 weeks due to the theobroma oil content. The low crystallinity of this matrix suggests its suitability for the formulation of nanostructured lipid carriers as high drug load could be reached because of the disorder in the matrix.

3.2. Wide angle X-ray diffraction (WAXD) studies

WAXD analysis of lipid matrices gives information on the crystalline state of the matrices, as it reveals the dimensions of the short spacings of the unit cells. Fig. 3 shows representative WAXD diffractogram of the lipid matrices measured after 12 weeks, while the WAXD data of the diffractograms measured after 1, 7, and 12 weeks are presented in Table 1. Theobroma oil had strong reflection at $2\theta = 19.3°$ $d = 4.60$ Å corresponding to the stable β modification which chain packing is triclinic [6,25–27]. Other reflections of medium and low intensities characteristic for theobroma oil occurred at $2\theta = 6.9°$ $d = 12.81$ Å, $2\theta = 16.3°$ $d = 5.44$ Å, $2\theta = 21.7°$ $d = 4.10$ Å, $2\theta = 22.0°$ $d = 4.04$ Å, $2\theta = 22.7°$ $d = 3.92$ Å, $2\theta = 23.0°$ $d = 3.87$ Å, and $2\theta = 24.0°$ $d = 3.71$ Å. These reflections remained unchanged after 7 and 12 weeks since the theobroma oil was in the stable form (Table 1). Beeswax on the other hand had high intensity reflections at $2\theta = 21.3°$ $d = 4.17$ Å, medium intensity reflection at $2\theta = 23.6°$ $d = 3.77$ Å, and low intensity reflections at $2\theta = 5.9°$ $d = 14.98$ Å and $2\theta = 19.2°$ $d = 4.62$ Å after 1 week (Table 1). These reflections remained more less unchanged after 7 and 12 weeks (Table 1), and are characteristic of stable form of beeswax. The reflections due to beeswax at $2\theta = 21.3°$ $d = 4.17$ Å and $2\theta = 23.6°$ $d = 3.77$ Å are typical of an orthorhombic subcell, and have been noted for beeswax [28]. The admixtures of theobroma oil and beeswax produced reflections which were intermediate between those of the pure substances. The strong intensity reflection due to theobroma oil ($2\theta = 19.3°$ $d = 4.60$ Å) was present in all the admixtures after 1 week (Table 1). The intensities, however, were proportional to the concentration of theobroma oil present in the lipid matrices. This high intensity reflection due to stable β modification of theobroma oil appeared after 7 and 12 weeks at the same positions. There was slight increase in intensity for lipid matrices containing 50% w/w and 75% w/w theobroma oil from initial values of 6939.94 counts (cts) and 9440.82 cts, respectively, after 1 week to 7215.56 cts and 10249.35 cts, respectively, after 12 weeks (not shown in Table 1), while there was a decrease in intensity of the reflection for the admixture containing 25% w/w theobroma oil from 3125.47 cts after 1 week to 1906.96 cts after 12 weeks. Also noticeable was the growth in the intensities of the reflections at $2\theta = 22.3°$ $d = 3.99$ Å and $2\theta = 22.9°$ $d = 3.88$ Å, which appeared in the admixtures containing 25% w/w and 75% w/w theobroma oil (Table 1). The main reflection due to beeswax at $2\theta = 21.3°$ $d = 4.17$ Å (or $2\theta = 21.4°$ $d = 4.15$ Å) also occurred almost at the same positions after 7 and 12 weeks in the lipid admixtures. However, the intensity decreased in the matrices containing 25% w/w and 50% w/w theobroma oil from 16725.28 cts and 17590.28 cts, respectively, after 1 week to 7543.50 cts and 14557.56 cts, respectively, after 12 weeks. These changes may suggest decrease in crystallinity

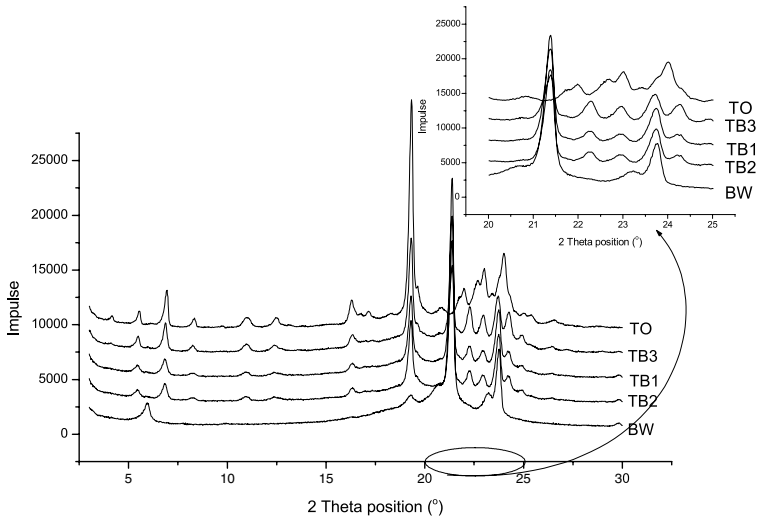

Fig. 3. WAXD diffractograms of the lipid matrices after 12 weeks: TO (theobroma oil), BW (beeswax), TB3 (75% w/w theobroma oil), TB1 (50% w/w theobroma oil), and TB2 (25% w/w theobroma oil). Inset shows the magnified portion of the diffractograms between 20° and 25°.

Table 1
WAXD data for the lipid matrices containing theobroma oil and beeswax at different stages of storage

TO						TB3						TB1						TB2						BW					
1 week		7 weeks		12 weeks		1 week		7 weeks		12 weeks		1 week		7 weeks		12 weeks		1 week		7 weeks		12 weeks		1 week		7 weeks		12 weeks	
2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)
4.2	21.04	4.2	21.04	4.2	21.04	4.0	22.09			4.0	22.09																		
5.5	16.07	5.5	16.07	5.6	15.78	*5.5*	*16.07*	5.5	16.07	5.5	16.07	5.5	16.07	5.4	16.36	5.5	16.07	5.4	16.36	5.5	16.07	5.4	16.36						
						6.0	14.73																	5.9	14.98	6.0	14.73	6.0	14.73
6.9	12.81	6.9	12.81	6.9	12.81	*6.9*	*12.81*	6.9	12.81	6.9	12.81	6.8	13.00	6.8	13.00	6.8	13.00	6.8	13.00	6.8	13.00	6.8	13.00						
8.3	10.65	8.3	10.65	8.3	10.65	8.3	10.65	8.2	10.78	8.3	10.65	8.2	10.78	8.2	10.78	8.2	10.78	8.2	10.78	8.2	10.78	8.2	10.78						
10.9	8.12	10.9	8.12	11.1	7.97	11.0	8.04	11.0	8.04	11.0	8.04	11.0	8.04	10.9	8.12	10.9	8.12	10.9	8.12	10.9	8.12	10.9	8.12						
12.5	7.08	12.5	7.08	12.5	7.08	12.4	7.14	12.4	7.14	12.4	7.14	12.4	7.14	12.3	7.20	12.4	7.14	12.5	7.08	12.5	7.08	12.3	7.20						
16.3	5.44	16.3	5.44	16.3	5.44	16.4	5.41	16.3	5.44	16.3	5.44	16.3	5.44	16.3	5.44	16.3	5.44	16.4	5.41	16.4	5.41	16.4	5.41						
17.2	5.16	17.1	5.19																										
																										17.7	5.01		
18.3	4.85	18.3	4.85																										
19.3	**4.60**	**19.3**	**4.60**	**19.3**	**4.60**	**19.3**	**4.60**	**19.3**	**4.60**	**19.3**	**4.60**	*19.3*	*4.60*	*19.3*	*4.60*	*19.3*	*4.60*	19.3	4.60	19.3	4.60	19.2	4.62	19.2	4.62	19.3	4.60	19.2	4.62
19.6	4.53	19.6	4.53																										
																								20.5	4.33	20.6	4.31	20.7	4.29
20.8	4.27	20.8	4.27	20.9	4.25																								
						21.4	**4.15**	**21.4**	**4.15**	**21.4**	**4.15**	**21.4**	**4.15**	**21.4**	**4.15**	**21.4**	**4.15**	**21.4**	**4.15**	**21.4**	**4.15**	**21.3**	**4.17**	**21.3**	**4.17**	**21.3**	**4.17**	**21.4**	**4.15**
21.7	4.10	21.7	4.10																										
22.0	4.04	22.0	4.04	22.0	4.04																								
						22.3	3.98	*22.3*	*3.98*	*22.3*	*3.98*	22.2	4.00	22.2	4.00	22.3	3.98	22.2	4.00	22.2	4.00	22.2	4.00						
22.7	3.92	22.7	3.92	22.7	3.92																								
23.0	*3.87*	*23.0*	*3.87*	23.0	3.87	22.9	3.88	*22.9*	*3.88*	*23.0*	*3.87*	22.9	3.88	22.9	3.88	23.0	3.88	22.9	3.88	22.9	3.88	22.9	3.88						
																								23.1	*3.85*	23.2	3.83	23.2	3.83
23.7	3.75					*23.7*	*3.75*	*23.8*	*3.74*	*23.7*	*3.75*	*23.7*	*3.75*	*23.7*	*3.75*	*23.8*	*3.74*	*23.7*	*3.74*	*23.7*	*3.75*	*23.7*	*3.75*	*23.6*	*3.77*	*23.7*	*3.75*	*23.8*	*3.74*
24.0	*3.71*	*24.0*	*3.71*	*24.0*	*3.71*																								
						24.2	*3.68*	*24.2*	*3.68*	*24.3*	*3.66*	24.2	3.68	24.2	3.68	24.2	3.68	24.2	3.68	24.2	3.68	24.2	3.68						
25.0	3.56	25.0	3.56	25.0	3.56	24.9	3.58	24.9	3.58	24.9	3.58	24.9	3.58	24.8	3.59	24.9	3.58												
25.4	3.51	25.3	3.52																										
26.5	3.36	26.5	3.36	26.6	3.35	26.4	3.38	26.4	3.38	26.5	3.36																		
28.7	3.11																												
						29.8	3.00	29.8	3.00			29.8	3.00	29.8	3.00	29.8	3.00	29.8	3.00	29.8	3.00	29.7	3.01	29.7	3.01	29.8	3.00	29.8	3.00

Key: high intensity (bold), medium intensity (italics), low intensity (underline) and weak intensity (normal). TO (theobroma oil), BW (beeswax), TB3 (75% w/w theobroma oil), TB1 (50% w/w theobroma oil) and TB2 (25% w/w theobroma oil).

with storage. However, the fact that there was increase in intensity of the reflections detected for the admixture containing 25% w/w theobroma oil at $2\theta = 6.8°$ $d = 13.00$ Å, $2\theta = 22.2°$ $d = 4.00$ Å, and $2\theta = 22.9°$ $d = 3.88$ Å may give a clue to its higher CI compared with other lipid admixtures.

The region between 20° and 25° was magnified (see inset) to have a closer observation of the reflections at that region. The reflections presented by the lipid matrix admixtures at $2\theta = 24.2°$ $d = 3.68$ Å were not characteristic of either beeswax or theobroma oil. They appear to be the result of modification of the lipid matrices due to interaction of the reflections due to theobroma oil and beeswax at $2\theta = 24.0°$ $d = 3.71$ Å and $2\theta = 23.6°$ $d = 3.77$ Å, respectively. This phenomenon also occurred at $2\theta = 22.3°$ and 22.2° where reflections not characteristic of either theobroma oil or beeswax were detected. These changes at the wide angle region may account for the observed differences in the lattice structure of the lipid matrix admixtures in terms of mixed crystals compared with the pure lipids. The characteristic reflections of theobroma oil in the admixtures showed some correlation with its content. However, beeswax did not show similar trend. This may be due to the fatty acid composition of beeswax. Beeswax contains mainly esters of higher fatty acids (up to C36) with some lower chain fatty acids (C16 and C18) which are present in theobroma oil. Mixtures of these lipids resulted in increase in the reflection intensity of theobroma oil as a result of increase in the amount of similar fatty acids present. Combination of theobroma oil and beeswax thus resulted in lipid matrices where small amounts of mixed crystals of either components form besides mixtures of crystals of the pure compounds. Presence of mixed crystals and mixture of crystals result in some disorder that increase drug incorporation and holding capacity of lipid matrices as drugs are entrapped in different sites within the lipid matrix. Lipid matrices containing 50% w/w and 75% w/w theobroma oil possess crystal characteristics that will favour drug incorporation. All the lipid matrices presented sharp reflections in the high angle region indicating that some of the hydrocarbon chains of the matrices are stiff and fully extended [29]. The reflections at $2\theta = 21.3°$ $d = 4.17$ Å and $2\theta = 23.6°$ $d = 3.77$ Å are probably due to crystalline lipids and are characteristically produced by crystalline lipids with an orthorhombic perpendicular alkyl chain packing arrangement [30,31]. We therefore, state that the two component lipid matrices exist as mixtures of the respective compound crystals besides some minor fractions of mixed crystals.

3.3. Small angle X-ray diffraction (SAXD) measurements

Many lipids are known to arrange themselves in layered structures with a repeat distance of few nanometers, thus giving rise to Bragg reflections in the small angle region. The repeat distances correspond to the thickness of the lipid bilayer. Theobroma oil had two prominent reflections at $2\theta = 1.3°$ $d = 67.96$ Å and $2\theta = 2.8°$ $d = 31.56$ Å after 1 week (Table 2). These reflections depict roughly a lamellar arrangement since they are the 1st and 2nd order reflections, respectively. Theobroma oil has been widely studied and its X-ray properties are well documented. This result is in consonance with reported findings [6].

The admixture containing 75% w/w theobroma oil had two prominent reflections at $2\theta = 1.3°$ $d = 67.96$ Å and $2\theta = 2.8°$ $d = 31.56$ Å. This arrangement also roughly depicts lamellar crystal arrangement [11]. Here, the influence of beeswax was only noticed by the broad nature of the reflection at $2\theta = 1.3°$. A weak reflection appeared after 7 weeks at $2\theta = 1.9°$ $d = 46.50$ Å, and the intensities of the reflections roughly remained unchanged after 12 weeks but the reflections shifted slightly to higher angles which may signify contraction of the bilayer structure (Table 2).

In the matrix consisting of 50% w/w theobroma oil, two high and broad reflections, and a weak reflection were detected after 1 week, respectively, at $2\theta = 0.9°$ $d = 98.17$ Å and $2\theta = 1.3°$ $d = 67.96$ Å, and $2\theta = 2.8°$ $d = 31.56$ Å. After 7 weeks, the reflection at $2\theta = 0.9°$ $d = 98.17$ Å was not detected. The two remaining reflections were detected at $2\theta = 1.2°$ $d = 73.63$ Å and $2\theta = 2.8°$ $d = 31.56$ Å after 12 weeks (Table 2, Fig. 4). With this result, the lipid admixture may have most of the matrices in 73.63 Å and small fraction in 31.56 Å bilayer arrangement.

The admixture containing 25% w/w theobroma oil, initially showed a high and broad reflection at $2\theta = 0.9°$ $d = 98.18$ Å and a very weak signal at $2\theta = 2.7°$ $d = 32.73$ Å after 1 week (Table 2). After 7 weeks, the high and broad reflection shifted to $2\theta = 1.0°$ $d = 88.35$ Å with the appearance of another low intensity reflection at $2\theta = 1.2°$ $d = 73.63$ Å, but the weak reflection at $2\theta = 2.7°$ $d = 32.73$ Å was not detected. However, after 12 weeks, the main broad reflection remained at $2\theta = 1.0°$ $d = 88.35$ Å with the disappearance of the low intensity reflection at $2\theta = 1.2°$ $d = 73.63$ Å and appearance of a weak reflection at $2\theta = 2.8°$ $d = 31.56$ Å. In this lipid matrix admixture, the preferable arrangement may be in 88.35 Å bilayer with a small fraction in 31.56 Å bilayer.

The very high intensity reflection of beeswax at $2\theta = 2.0°$ $d = 44.18$ Å was conspicuously missing in all the binary lipid matrices in SAXD region, although theobroma oil itself reveals a weak interference at this position (Table 2, Fig. 4). From this finding an internalization of beeswax into the theobroma oil bilayer may be concluded which accounts for the use in stiffening theobroma oil suppository formulations, a practice which has been in use for ages. These lipid admixtures could thus provide alternatives to single lipids with either low crystallinity that cannot withstand ambient temperature especially in the tropics or lipids of very high crystallinity which have been found to exclude the

Table 2
SAXD data for the lipid matrices containing theobroma oil and beeswax at different stages of storage

TO					TB3						TB1						TB2						BW					
1 week		7 weeks		12 weeks			1 week		7 weeks		12 weeks		1 week		7 weeks		12 weeks		1 week		7 weeks		12 weeks		1 week		7 weeks		12 weeks	
2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)		2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)
													0.9	*98.18*					**0.9**	**98.18**	1.0	88.35	*1.0*	*88.35*	**0.9**	**98.18**	**0.9**	**98.18**	0.9	98.18
							1.2	*73.63*									1.2	73.63			1.2	73.63								
1.3	**67.96**	**1.4**	**63.11**	**1.4**	**63.11**		*1.3*	*67.96*	1.3	67.96	*1.4*	*63.11*	*1.3*	*67.96*	1.3	67.96														
2.0	44.17	2.2	40.16	2.1	42.07				1.9	46.50	2.1	42.07													**2.0**	**44.18**	**2.0**	**44.18**	**2.0**	**44.18**
2.8	*31.56*	*2.8*	*31.56*	*2.9*	*30.46*		*2.8*	*31.56*	2.8	31.56	*2.9*	*30.46*	2.8	31.56	2.8	31.34	2.8	31.56	2.7	32.73			2.8	31.56						

Key: high intensity (bold), medium intensity (italics), low intensity (underline) and weak intensity (normal). TO (theobroma oil), BW (beeswax), TB3 (75% w/w theobroma oil), TB1 (50% w/w theobroma oil) and TB2 (25% w/w theobroma oil).

Fig. 4. SAXD diffractograms of the lipid matrices after 12 weeks: TO (theobroma oil), BW (beeswax), TB3 (75% w/w theobroma oil), TB1 (50% w/w theobroma oil) and TB2 (25% w/w theobroma oil).

incorporated drug during recrystallization [32]. All the two component lipid matrices were found to be roughly lamellar thus highlighting the fact that the crystal arrangement of the individual lipids was not greatly disorganized. Lipid matrices with a certain degree of disorder are considered to be ideal for formulation of nanoparticulate lipid carriers due to their high active ingredient payload capacity. In that case, especially the admixture containing 75% w/w theobroma oil may be recommended for such application. Rational combination of beeswax and theobroma oil could provide an array of lipid matrices for the drug formulator considering the increasing interest in lipid drug delivery system and the natural origin of these two lipids. Natural lipids have been shown to be better tolerated than semi-synthetic lipids [3].

3.4. Polarized light microscopy (PLM)

Crystalline and liquid crystalline structures often show typical optical textures when viewed in crossed polarized light. The PLM micrographic structures are presented in Fig. 5. The micrographs showed greater crystal growth with storage in the lipid matrices except pure beeswax and the admixture with high amount of beeswax. The lipid matrix containing 75% w/w theobroma oil showed spherulite structure after 4 weeks. These radial lamellar structures are just crystal aggregates as they did not present any other distinctive interference on either SAXD or WAXD even after 12 weeks. There was no increase in reflection intensity signifying increase in crystallinity. Such structures may

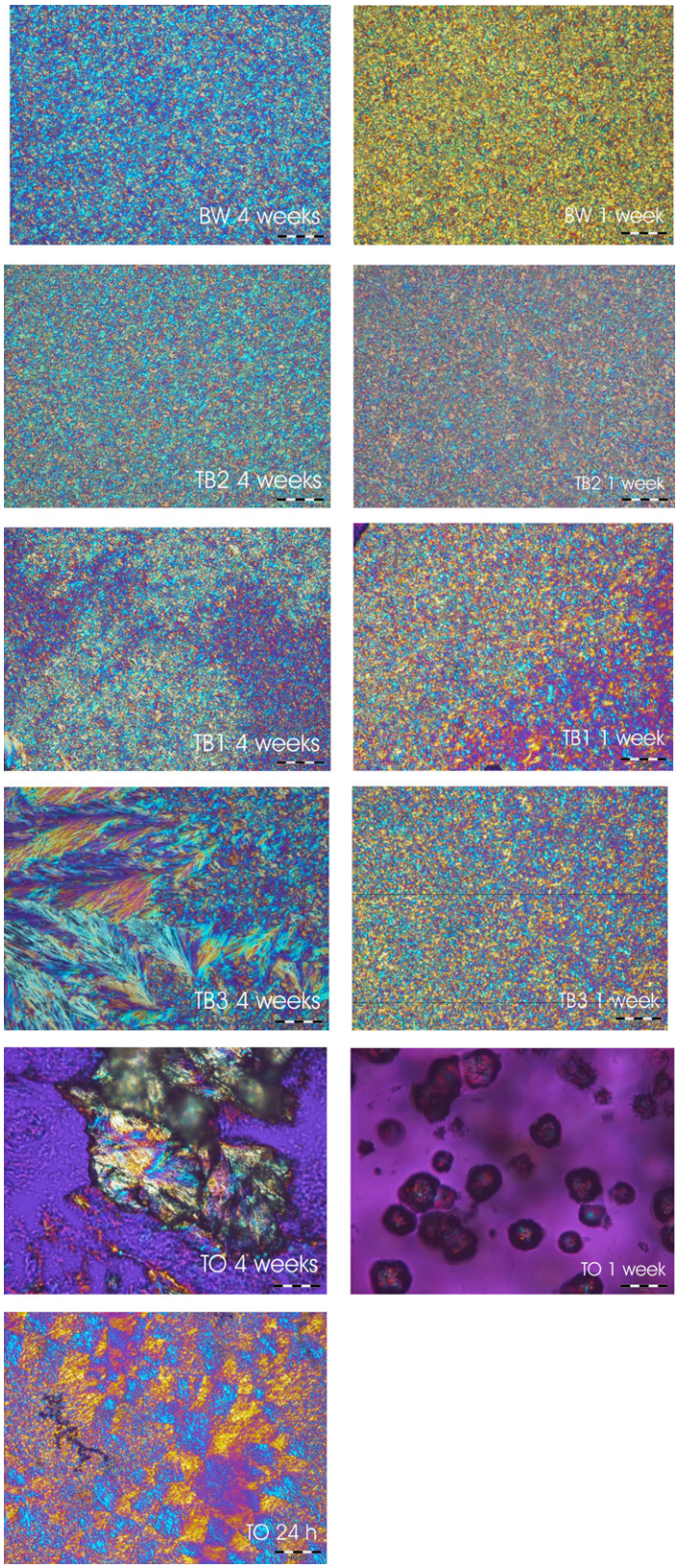

Fig. 5. Polarized light micrographs of some of the lipid matrices after 24 h, 1 week, and 4 weeks: TO (theobroma oil), BW (beeswax), TB3 (75% w/w theobroma oil), TB1 (50% w/w theobroma oil), and TB2 (25% w/w theobroma oil). Bar represents 100 μm.

have influence on drug incorporation but it should be noted that PLM observation was done under static condition without influence of any additive. In the 50% w/w theobroma oil-containing admixture, there was a noticeable non-uniformity in crystallization especially after 1 and 4 weeks, although it appeared uniform after 24 h. During

crystallization, the material goes through different stages beginning with nucleation, and followed by unhindered growth and structure formation. The initial nucleation is characterized by the appearance of nuclei far apart from each other. This non-uniform crystallization may result in mixture of crystals and low crystal order which may increase drug loading [32]. There was more crystal growth in this 50% w/w admixture as evidenced by their PLM micrographs after 1 and 4 weeks (Fig. 5). Theobroma oil alone showed evidence of coarser structures after 24 h. Discrete crystals emerged after 1 week which further aggregated to bigger crystals after 4 weeks compared with beeswax. This may coincide with β' to β polymorphic transition and is in perfect agreement with reported works [24,33]. The phase transition from β' form to β polymorph usually leads to the formation of large microstructures. No characteristic mesophases were observed as these matrices passed from the crystalline state to isotropic state upon heating and vice versa upon cooling.

3.5. Freeze-fracture transmission electron microscopy (FFTEM)

The FFTEM micrographs are presented in Fig. 6. FFTEM revealed characteristic lamellar packing of the lipid matrices. Beeswax showed a more uniform lamellar packing with some extended layers than either theobroma oil or the 1:1 combination with beeswax. The regular and sharp edges of some lamellar areas are typical of crystalline state [34]. The micrograph of the lipid matrix containing 50% w/w theobroma oil in addition showed some surface irregularities which is in agreement with a reduced degree of order.

3.6. Isothermal heat conduction microcalorimetry (IMC)

Microcalorimetry is an analytical technique that has found numerous applications within the pharmaceutical environment especially in solid state pharmaceutics such

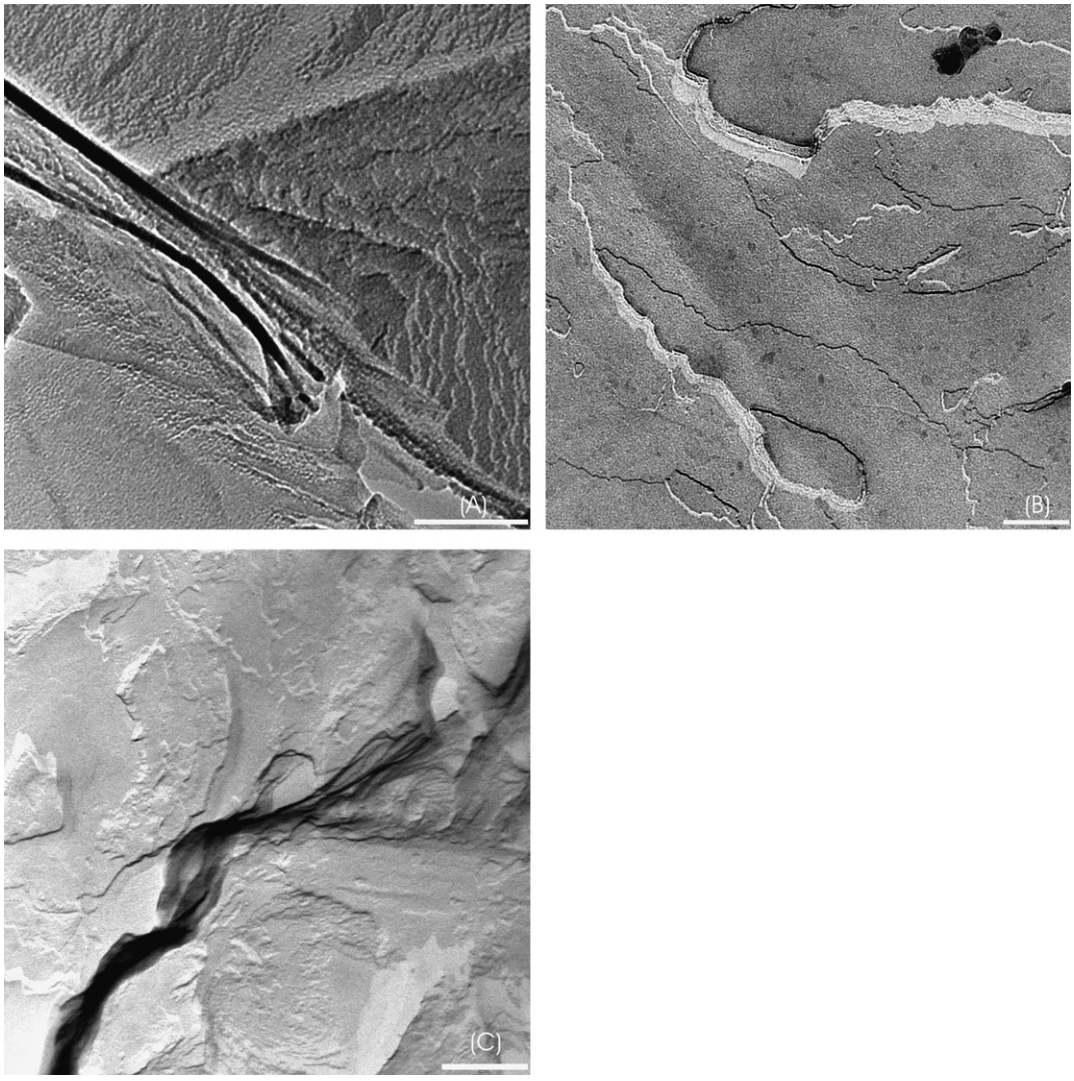

Fig. 6. Freeze-fracture transmission electron micrographs of the lipid matrices: theobroma oil (A), beeswax (B) and 50% w/w theobroma oil containing admixture (C). Bars represent 100, 200, and 200 nm, respectively.

as physical form characterization. Isothermal microcalorimetry provides precise and rapid knowledge about possible solid state transition processes. Preformulation issues such as amorphicity and polymorphism can be assessed with ease, precision, and confidence. The result of IMC measurement is presented in Fig. 7. The figure shows that addition of 25% w/w beeswax to theobroma oil produced no change in heat flow over time and thus no change in crystallization behavior in the resulting lipid matrix. The crystallization process was spontaneous indicated by the high negative slope [12]. However, the lipid matrix containing 50% w/w beeswax had a short delay in crystallization shown by the early segment of the crystallization exotherm with positive slope [12]. There was also a further slight event of heat flow in this lipid matrix within the first 45 min of isothermal crystallization indicated by a very slight change progress of the crystallization, which may just be an overshoot of the initial positive slope detected. A closer look at the exotherms shows that compared with the lipid matrix containing 25% w/w theobroma oil and beeswax, there was greater heat flow in theobroma oil, 50% w/w and 75% w/w theobroma oil-containing lipid matrices, signifying that beeswax affected the heat flow significantly only at 75% w/w concentration. The lipid matrix containing 25% w/w theobroma oil with very low heat flow indicated a delay in crystallization after an initial spontaneous crystallization which obviously had already occurred before starting the measurement. This delay in crystallization together with the initial spontaneous crystallization contributes to the high crystallinity index of this matrix compared to the other lipid matrices. Lipid matrices that crystallize slowly lead to higher degree of crystal order. All the IMC curves became asymptotic to time axis implying that ultimate crystallinity would be reached at infinite time. IMC predicts ease of crystallization of lipid matrices after melting. These results show that with 25% w/w theobroma oil in beeswax, a long delay in recrystallization should be expected by the formulator compared with 50% w/w and 75% w/w concentrations. This result also guides the formulator in drug content determination as analysis done before complete recrystallization of the system may lead to false high value of drug encapsulation efficiency.

Eq. (3) was further analyzed within the first 720 s of spontaneous isothermal crystallization to obtain the crystallization kinetic parameters. The normalized plot for Eq. (3) is presented in Fig. 8, while the kinetic parameters are presented in Table 3. The early stage of the overall crystallization from a supercooled melt obeys Avrami equation very well. However, depending on experimental conditions, deviations usually occur from Avrami models. For instance, the Avrami exponent n is usually a positive integer between 1 and 4, but fractional values of the exponent and deviation from Avrami equation at later stage are generally attributed to the assumptions made in the Avrami model such as constant radial growth, constant density and shape of the growing nuclei, uniqueness of nucleation and no volume change during phase transformation [13,35]. Although high correlation coefficients ($r^2 > 0.9$) were obtained from the evaluation of the crystallization kinetics of the lipid matrices from the melts, the Avrami exponents obtained were not integers (Table 3). Low non-integral values were obtained for all the lipid matrices. High values ($n = 4$) could result from three dimensional

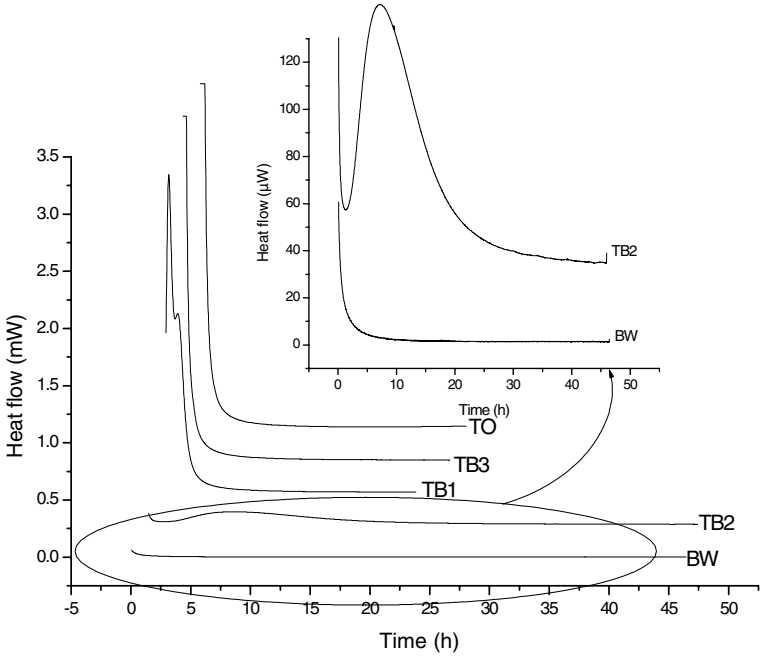

Fig. 7. Crystallization exotherms of the lipid matrices: TO (theobroma oil), BW (beeswax), TB3 (75% w/w theobroma oil), TB1 (50% w/w theobroma oil), and TB2 (25% w/w theobroma oil).

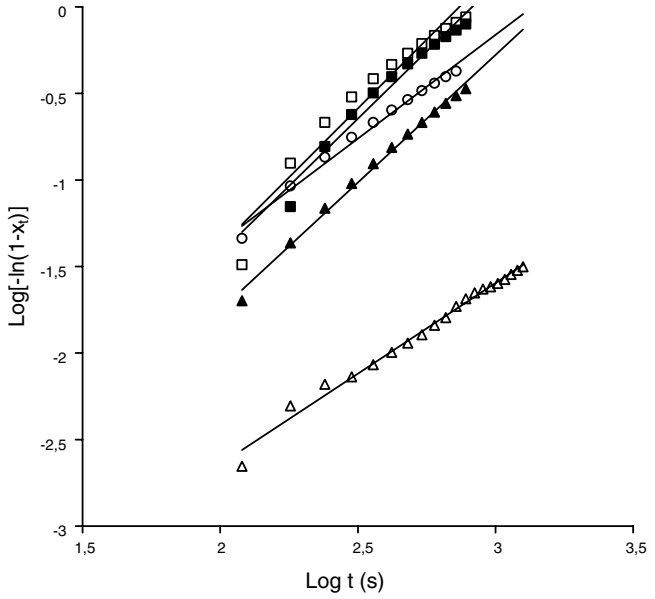

Fig. 8. Isothermal crystallization kinetic plots for theobroma oil/beeswax lipid matrices: ■ theobroma oil, ○ beeswax, □ 75% w/w theobroma oil, ▲ 50% w/w theobroma oil, Δ 25% w/w theobroma oil.

Table 3
Crystallization kinetic parameters within the first 720 s of spontaneous isothermal crystallization

Lipid matrix	Parameter		
	n	k (s^{-1})	r^2
TO	1.555	2.92×10^{-5}	0.9668
TB3	1.591	2.73×10^{-5}	0.9420
TB1	1.474	2.00×10^{-5}	0.9942
TB2	1.047	1.83×10^{-5}	0.9883
BW	1.199	17.40×10^{-5}	0.9871

TO (theobroma oil), BW (beeswax), TB3 (75% w/w theobroma oil), TB1 (50% w/w theobroma oil) and TB2 (25% w/w theobroma oil).

growth from homogeneous nuclei, simultaneous growth of two different types of structures or a single structure grown from two types of nuclei: homogeneous and heterogeneous [13]. The low values obtained here may indicate heterogeneous nucleation or that the nucleation mechanisms were more instantaneous in time [36]. On looking at the growth rate constants (k values), beeswax had the highest rate constant indicating faster crystallization, but the admixtures had k and n values which increased almost in proportion to theobroma oil concentration. This indicates that theobroma oil with lower melting point had a strong influence on both the nucleation mechanism and crystal growth. Theobroma oil and lipid matrices containing 50% w/w and 75% w/w theobroma oil with close n values may possess similar nucleation mechanisms but different growth rates, as may be applicable to beeswax and the lipid matrix containing 25% w/w theobroma oil. The influence of theobroma oil could also be appreciated from the fact that lipid matrix containing greater amount of beeswax had a lower n value compared to beeswax alone because of the influence of theobroma oil. The delay in crystallization of the lipid matrix containing 25% w/w theobroma oil (Fig. 7) may be due to its very low n and k values compared to other admixtures, thus a possible difficulty in nucleation and subsequent growth cannot be ruled out. This result further lends proof to the fact that IMC studies could effectively address the question of how the crystallinity of one component affects the crystallization behavior of the other, at least in a two-component lipid mixture.

4. Conclusions

Mixtures of theobroma oil and beeswax formed matrices composed of mixtures of crystals, with the structure of which consist of small fractions of mixed crystals different from those formed by the pure lipids. DSC, PLM, XRD, and IMC studies suggested some of the two component lipid matrices were less ordered arrangement of crystals and this may be favourable for increasing drug loading capacity. Crystallization kinetics from IMC studies indicated that theobroma oil exerted a strong influence on both nucleation and crystal growth with the admixture containing 25% w/w theobroma oil possessing long delay in crystallization compared with others. Based on the result of this investigation, admixtures containing 50% w/w and 75% w/w theobroma oil in beeswax were selected for use in the formulation of solid lipid nanoparticles and nanostructured lipid carriers. On the basis of thermal and solid–liquid behaviours of these mixtures, they can be further explored through experimental research for the production of ideal lipid matrices for improved use in the formulation of nano- and microparticulate and solid self-emulsifying drug delivery systems for topical and systemic applications.

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