European Journal of Biotechnology and Bioscience
ISSN: 2321-9122; Impact Factor: RJIF 5.44
Received: 04-04-2019; Accepted: 07-05-2019
www.biosciencejournals.com
Volume 7; Issue 4; July 2019; Page No. 10-21

Formulation and optimization of biodegradable insulin loaded nanoparticles

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Abstract
This research aims to provide the diabetic patients with more adequate treatment by designing controlled release insulin loaded nanoparticles prepared by biodegradable polymers. Factors to be studied in this research are the effect of the preparation methods using two different preparation techniques microfluidic / salting out technique which is a new and promising technique and a conventional technique which is double emulsion /solvent evaporation technique. The characterization of the nanoparticles was performed in the terms of size, zeta potential, morphological analysis, IR spectrophotometry, thermal analysis, loading and *In vitro* release of the nanoparticles. All the formulations are showing nanoparticles in the range from 300 to 400 nm with a negative zeta potential between – 10 and – 20 mV. Furthermore, all the nanoparticles are showing smooth spherical shape with chemical and thermal compatibility between the ingredients with encapsulation efficiency that ranges from 42 % to 52 % in all formulations.

Keywords: microfluidic, salting out, emulsion, solvent evaporation

1. Introduction

Diabetes mellitus is considered one of the most common and serious diseases in the world which affected about 8.5 % of the adult world population in 2014. Most of the diabetic patients depend on multiple daily insulin injections for the treatment which is inconvenient to use for most of them. This research aims to design controlled release insulin loaded nanoparticles prepared by biocompatible and biodegradable polymers in order to provide the diabetic patients with adequate treatment as a substitute for the daily injection of insulin by studying different factors in the preparation process of the nanoparticles and how they affect the physicochemical properties of the nanoparticles [1].

The first factor to be discussed is the preparation method. Two techniques have been used in the manufacturing of the insulin loaded nanoparticles. The first technique is double emulsion/solvent evaporation technique which is a conventional method that is being used in the preparation of micro and nanoparticles for a long time. Also, this technique is being used in some industries on a large scale. A modified type of this technique has been used in this research which consists of two sequential emulsification steps each includes high homogenization force [2]. The first emulsification step is to prepare a primary emulsion w/o in which the insulin is solubilized in the aqueous phase and entrapped inside the oily phase which contains the polymers solubilized in dichloromethane. The second emulsification step is to prepare a w/o/w emulsion by homogenizing the primary emulsion in aqueous phase containing poly vinyl alcohol (PVA) as a stabilizer. The organic solvent used in the oily phase dichloromethane (DCM) which is a toxic volatile organic solvent used to solubilize the polymers is then removed by stirring the emulsion for one day allowing the organic solvent to be evaporated [2].

The second technique is microfluidic/salting out which is a new and promising technique has been recently used in the preparation of nanoparticles. Furthermore, microfluidic devices are simple and easy to use with a better control on the size and poly dispersity (PDI) of the nanoparticles produced [3]. Microfluidic technique includes only one step for the preparation of the nanoparticles and allows the control on many parameters. The insulin is solubilized in aqueous phase containing PVA as a stabilizer and the polymers solubilized in acetonitrile as an organic phase are introduced to the microfluidic device at controlled flow rate to produce the nanoparticles only in one step using the minimum amount of acetonitrile which is a toxic organic solvent. The organic solvent is then removed by salting out using sodium borate salt [4, 5]. The second factor that has been investigated in this research is the type of polymer used in the preparation of the insulin loaded nanoparticles.

2. Materials and Methods

2.1 Materials

Recombinant human insulin was purchased from (Sigma-Aldrich, UK) with a molecular weight of 5800 Da. PLGA Resomer® RG 503 H, Poly(D, L-lactide-co-glycolide) acid terminated with lactide: glycolide ratio 50:50 and molecular weight of 24,000 – 38,000 Da, Poly (DL-lactide-co-caprolactone) with a ratio of 85:15 lactide: caprolactone DL-lactide 86 %, Poloxamer 188 solution 10 % and Poly(vinyl alcohol) with molecular weight of 13,000 – 23,000 Da were also purchased from (Sigma-Aldrich, UK). Table (1) shows the prepared formulations and composition.

Table 1: Table showing different types of formulations manufactured by different polymers and techniques

Formulation ID	Technique used	Composition (mg)			
		PLGA Resomer® RG 503 H	Poly (D, L-lactide-co-caprolactone)	poloxamer 188	Insulin
S1 DE	Double emulsion / solvent evaporation	200			4
S2 DE		200			4
S3 DE		200			4
S4 DE		190	10		4
S5 DE		190	10		4
S6 DE		190	10		4
S7 DE		180	10	10	4
S8 DE		180	10	10	4
S9 DE		180	10	10	4
S1 MF	Microfluidic /salting out	200			4
S2 MF		200			4
S3 MF		200			4
S4 MF		190	10		4
S5 MF		190	10		4
S6 MF		190	10		4
S7 MF		180	10	10	4
S8 MF		180	10	10	4
S9 MF		180	10	10	4

2.2 Methodology

2.2.1 Preparation of insulin-loaded nanoparticles using double emulsion / solvent evaporation technique

A modified double emulsion technique has been used in this method which includes preparing a primary dispersion of the drug in organic phase then further emulsification to incorporate the dispersed particles into bigger particles as w/o/w as shown in figure (1).

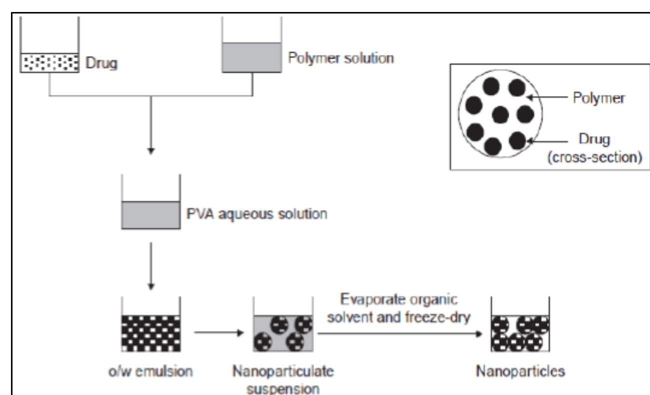

Fig 1: Showing a diagram for double emulsion technique [6].

As shown in figure (2) The aqueous phase was prepared by dissolving 4 mg of insulin in 0.5 ml of 0.1 M HCl (as insulin has better solubility at lower pH 2-2.5 pH) in Eppendorf tube then 0.5 ml of 2.5 % PVA was added with shaking with vortex (whirlimixer, fisher scientific) till complete dissolving of insulin. For the organic phase 200 mg of polymers as shown in figure (1) were added to 6 ml of dichloromethane with stirring till complete dissolving of the polymers. The primary emulsification was performed by adding the aqueous phase drop wise to the organic phase and homogenization at 6000 rpm for 2 minutes. Then the secondary emulsification was performed adding the primary (W/O) emulsion to 50 ml of 1.25 % PVA as external phase while homogenization at 10000 rpm for 6 minutes using (Silverson homogenizer, UK) to form (W/O/W) emulsion. Then evaporation of dichloromethane by stirring the system for 24 hrs. After that centrifugation for 15 minutes at 6000 rpm using centrifuge (MSE mistral 1000, UK) and the water was removed then washed for three times to remove the residues of the organic solvent. After that samples were freeze dried using freeze dryer (VirTis BenchTop K series, USA).

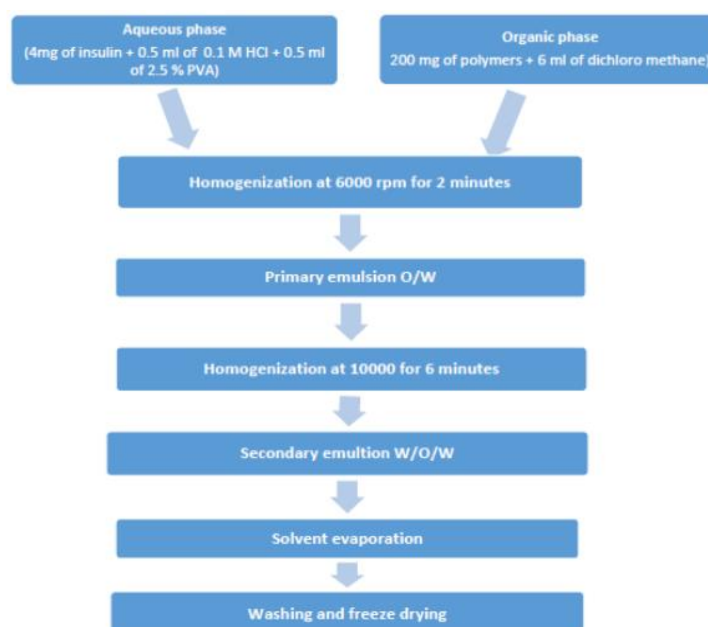

Fig 2: Flow chart showing manufacturing steps of insulin loaded nanoparticles using double emulsion technique

2.2.2 Preparation of insulin-loaded nanoparticles using microfluidic technique

The aqueous phase was prepared by weighing 4 mg of insulin in Eppendorf tube then 0.5 ml of 0.1 M HCl was added with shaking using vortex mixer (Whirlimixer, fisher scientific, UK) till complete dissolution of the insulin. Then, 0.5 ml of 2.5 % PVA solution was added and mixed using vortex mixer to make sure insulin is completely dissolved. For the organic phase, 4 ml of acetonitrile were added to 200 mg of the polymers in a beaker while stirring using magnetic stirrer to dissolve the polymers. After that, NanoAssemblr Benchtop instrument (Precision NanoSystems, Inc., Vancouver, BC, Canada) was used to manufacture the nanoparticles with TFR (total flow rate) of 12 ml/min which controls the mixing speed of the two phases in the microfluidic cartridge, and FRR (flow rate ratio) of 4:1 organic to aqueous phase as shown in figure (3). The resulting nanoparticles were stirred with sodium borate salt to remove acetonitrile. Then our system was centrifuged using centrifuge (MSE mistral 1000, UK) and the water was removed then washed to make sure all residues specially the organic solvent are completely removed. After that the nanoparticles were freeze dried using (VirTis BenchTop K series, USA) [7].

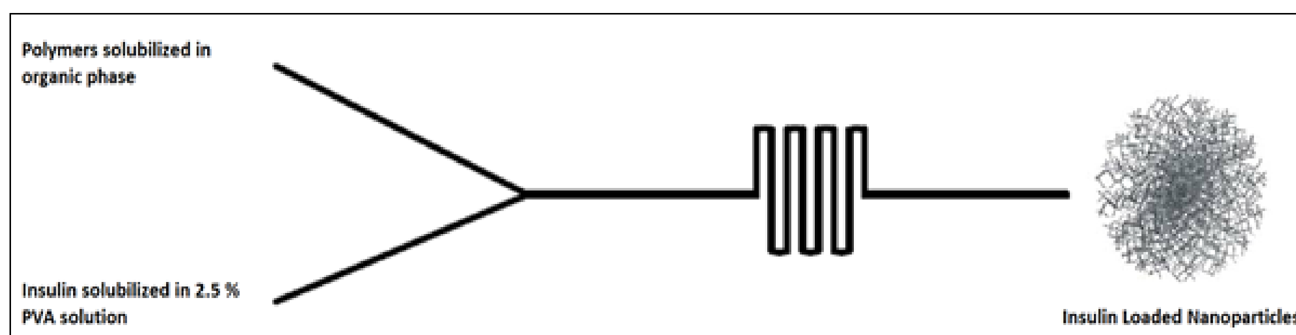

Fig 3: Showing the preparation process of the insulin loaded nanoparticles by microfluidic technique

2.2.3 Particle size characterization

The principle of this technique is fine particles are in constant random Brownian motion, the speed of the particles is related to the particle size at constant temperature. Small particles diffuse faster than large ones. Illuminating the particles produced by the speckle pattern with a laser is observed to measure the speed of the diffusion. By time the intensity of the scattering will fluctuate. Which can be detected by avalanche photodiode detector (APD). The changes in the intensity are then analysed with a digital autocorrelator that produces a correlation function. Then the curve produced can be analysed to give the size and size distribution shown in figure (4). Zetasizer Nano ZS® is easy to use and highly automated with high sensitivity. In addition, samples for size can be measured with little or no dilution [8].

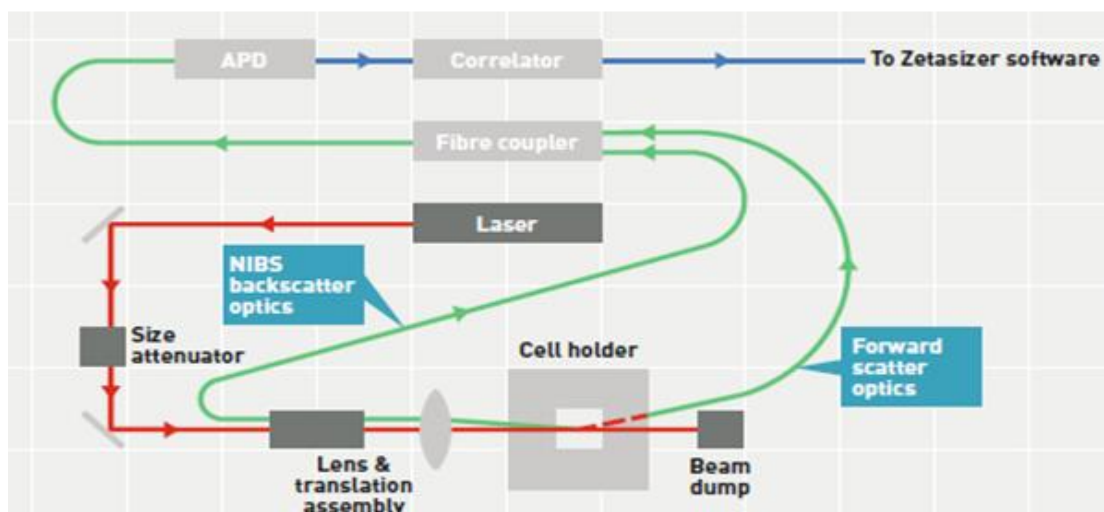

Fig 4: Diagram showing the theory of Zetasizer Nano and how it works

2.2.4 Zeta potential

Zeta potential was measured using Zetasizer Nano ZS® (Malvern Instruments Limited, UK). Samples were taken from each formula before the freeze drying step then diluted using 0.01 M KCl solution for better conductivity. Zeta potential is measured by applying an electric field to a particles dispersion or a solution then the velocity of the particles is then measured by applying a patented laser interferometric technique calls M3-PALS (phase analysis light scattering) which enables to calculate the electrophoretic mobility from which zeta potential can be measured [9].

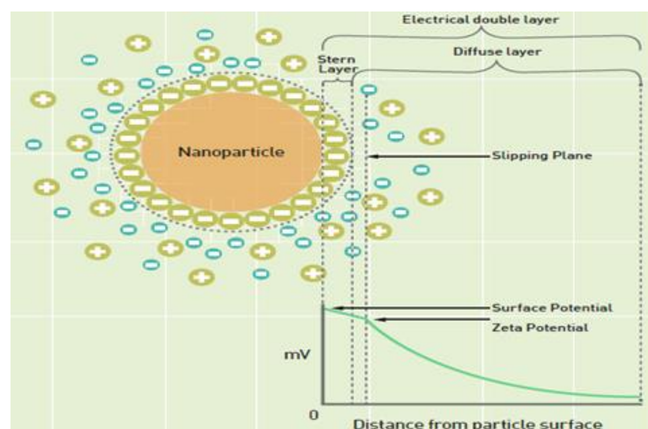

Fig 5: Showing the double layer surrounding a nanoparticle in a given medium

2.2.5 Morphological analysis and imaging

Imaging was performed using scanning electron microscope (SEM). Freeze dried samples were coated with a mixture of Au/Pd using (Quorum SC7620 Sputter Coater) to improve the image quality. Then imaging was performed using scanning electron microscope SEM (S-3000N, Hitachi, Japan) which can take images for the samples up to 50,000 x.

2.2.6 Loading of insulin and encapsulation efficiency

Insulin loading was performed using direct extraction method of insulin from the nanoparticles. 5 mg of insulin were weighed in Eppendorf tube then 0.5 ml of 1 M NaOH and incubated at 37°C for 24 hours to destroy the nanoparticles and allow the release of the encapsulated insulin. Then, samples were neutralized by 1 M HCl and centrifuged for 5 minutes at 10000 rpm then supernatant withdrawn for analysis and treated by Pierce™ BCA Protein Assay Kit which is a detergent-compatible formulation based on bicinchoninic acid (BCA) used as a calorimetric method to detect and quantify the total protein. This method is a combination between reduction of Cu+2 to Cu+1 by protein in the alkaline medium with the highly selective and sensitive detection of Cu+1 cation by the use of a reagent that contains bicinchoninic acid. This reaction produces a purple color that results from the chelation of one cuprous ion with two molecules of BCA. The water-soluble complex produced has a high absorbance at 562 nm and gives almost a linear line by increasing the concentration of the protein in the working range from 20 to 2000 µg/mL.

2.2.7 *In vitro* release of insulin loaded nanoparticles

In vitro release test was performed by adding 5 mg of insulin into 1 ml phosphate buffer (PBS pH 7.4) and incubated at 37 °C. Samples were withdrawn at predetermined time intervals 2 hours, 1 day, 2 days, 3 days, 4 days and 7 days. Three samples were withdrawn from each formula and replaced with fresh phosphate buffer. Samples withdrawn were analyzed by Pierce™ BCA Protein Assay Kit.

2.2.8 Compatibility and stability of insulin loaded nanoparticles

Stability and compatibility were assessed by two different methods. The first method is FTIR (Fourier transform infrared spectrophotometry) to assess the chemical stability and the second method is DSC (Differential scanning calorimeter) to assess the thermal behaviour and stability of the nanoparticles which will be discussed as follow.

2.2.9 FTIR (Fourier transform infrared spectrophotometry)

Chemical stability and compatibility of freeze dried samples of insulin loaded nanoparticles were measured using Fourier transform infrared spectrophotometer (IRAffinity-1S, Shimadzu, Japan). FTIR can detect the functional groups in the chemical substances qualitatively and quantitively. Furthermore, by comparing the spectrum of the samples with their raw materials we can detect any chemical reactions that might happens inside the nanoparticles and assess the compatibility of them.

2.2.10 DSC (Differential scanning calorimeter).

Stability and compatibility were also performed using differential scanning calorimeter (DSC q1000, TA instruments, UK). DSC was used to determine the transition temperature for each formula and its raw materials and compared together. Any change in the transition temperature of the samples over the raw materials indicates incompatibility or instability of the nanoparticles.

3. Results

The aim of research was to investigate the effect of manufacturing technique and polymer type on physiochemical properties of insulin loaded nanoparticles e.g. size, zeta potential, shape, stability, encapsulation efficiency and release of insulin.

3.1 Size

For the comparison between different techniques, nanoparticles prepared by the microfluidic technique exhibited smaller size than nanoparticles prepared by double emulsion technique for each formulation. Furthermore, all nanoparticles are showing small PDI (polydispersity index) that ranges from 0.15 to 0.35 which indicates low variation in the particle size and homogenous distribution (tables 2, 3 & fig.6).

Table 2: Particle size and PDI of insulin loaded nanoparticles performed by double emulsion /solvent evaporation technique

Double emulsion / solvent evaporation Technique					
Formulation ID	Size n.m	PDI	Polymer type	size nm (mean)	PDI avg
S1 DE	330	0.14	PLGA NPs	343 ± 33	0.22
S2 DE	357	0.29			
S3 DE	340	0.23			
S4 DE	310	0.12	PLGA NPs + 5% caprolactone copolymer	339 ± 28	0.15
S5 DE	365	0.2			
S6 DE	317	0.12			
S7 DE	320	0.22	PLGA NPs + 5% caprolactone copolymer+ 5 % poloxamer 188	328 ± 38	0.19
S8 DE	320	0.19			
S9 DE	353	0.16			

Table 3: Particle size and PDI of insulin loaded nanoparticles performed by microfluidic /salting out technique

Microfluidic / salting out technique					
Formulation ID	Size n.m	PDI	Polymer type	Size nm (mean)	PDI avg
S1 MF	281	0.20	PLGA NPs	328 ± 39	0.26
S2 MF	366	0.27			
S3 MF	337	0.31			
S4 MF	307	0.24	PLGA NPs + 5% caprolactone copolymer	321 ± 27	0.26
S5 MF	336	0.27			
S6 MF	351	0.29			
S7 MF	268	0.14	PLGA NPs + 5% caprolactone copolymer+ 5 % poloxamer 188	317 ± 59	0.31
S8 MF	332	0.44			
S9 MF	379	0.35			

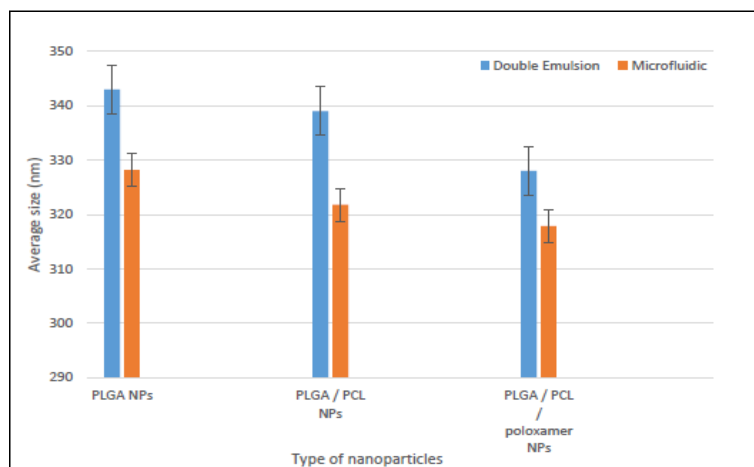

Fig 6: Graph showing results for average size of insulin loaded nanoparticles performed by both double emulsion / solvent evaporation and microfluidic / salting out technique

3.2 Zeta potential

For the nanoparticles prepared by microfluidic technique the nanoparticles prepared by PLGA with caprolactone copolymer and poloxamer 188 show the highest value of zeta potential with an average of -18.1 mV followed by PLGA nanoparticles and PLGA with caprolactone nanoparticles with average values of -13.2 mv and -12.13 mV respectively (table 4, 5 & fig.7).

Table 4: Table showing results for zeta potential of insulin loaded nanoparticles performed by double emulsion /solvent evaporation technique

Double emulsion / solvent evaporation technique				
Formulation ID	Z.P. mV	Polymer type	Z.P. avg mV	Standard Deviation
S1 DE	-11.6	PLGA NPs	-10.6	1.3
S2 DE	-9.0			
S3 DE	-11.3			
S4 DE	-10.2	PLGA NPs + 5% caprolactone copolymer	-11.7	1.8
S5 DE	-13.1			
S6 DE	-11.9			
S7 DE	-11.3	PLGA NPs + 5% caprolactone copolymer + 5 % poloxamer 188	-8.1	2.4
S8 DE	-6.0			
S9 DE	-6.9			

Table 5: Table showing results for zeta potential of insulin loaded nanoparticles performed by microfluidic /salting out technique

Microfluidic / salting out technique				
Sample Name	Z.P. mV	Polymer type	Z.P. avg mV	Standard Deviation
S1 MF	-15.8	PLGA NPs	-13.2	3.2
S2 MF	-9.2			
S3 MF	-14.6			
S4 MF	-12.1	PLGA NPs + 5 % caprolactone copolymer	-12.1	1.3
S5 MF	-11.9			
S6 MF	-12.5			
S7 MF	-16.6	PLGA NPs + 5 % caprolactone copolymer + 5 % poloxamer 188	-14.8	4.4
S8 MF	-9.2			
S9 MF	-18.7			

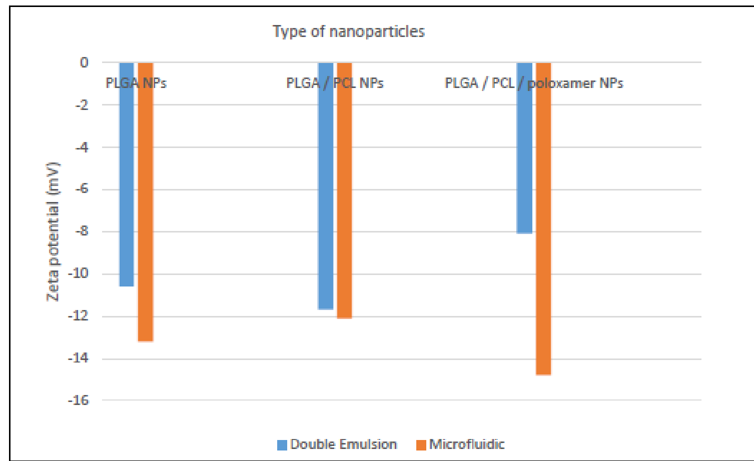

Fig 7: Graph showing results for average zeta potential of insulin loaded nanoparticles performed by both double emulsion / solvent evaporation and microfluidic / salting out technique

On the other hand, nanoparticles prepared by microfluidic technique are showing higher values of zeta potential than those prepared by double emulsion technique for each formulation.

3.3 Morphological analysis and imaging.

Nanoparticles prepared by double emulsion technique are showing slightly bigger particles and more variation in size as shown in figure (8,10,12) comparing to those prepared by microfluidic technique.

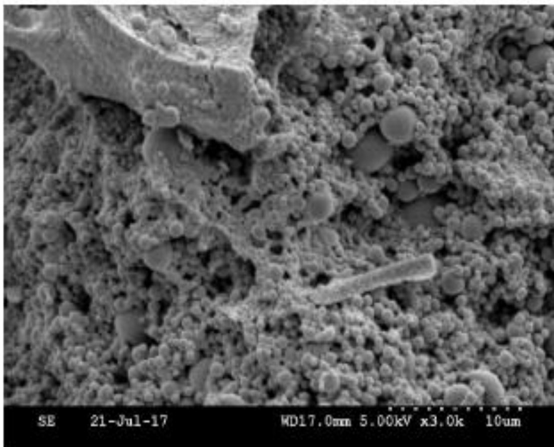

Fig 8: Showing SEM image fin PLGA nanoparticles prepared by double emulsion technique

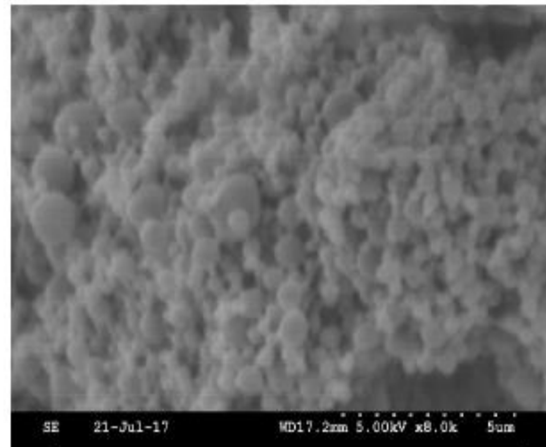

Fig 9: Showing SEM image for PLGA nanoparticles prepared by microfluidic technique

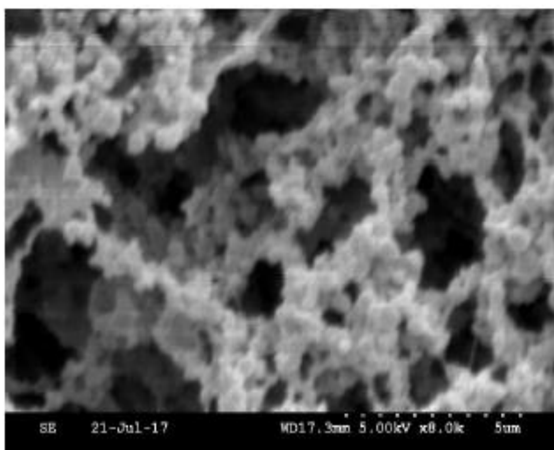

Fig 10: Showing SEM image for PLGA / caprolactone nanoparticles prepared by double emulsion technique

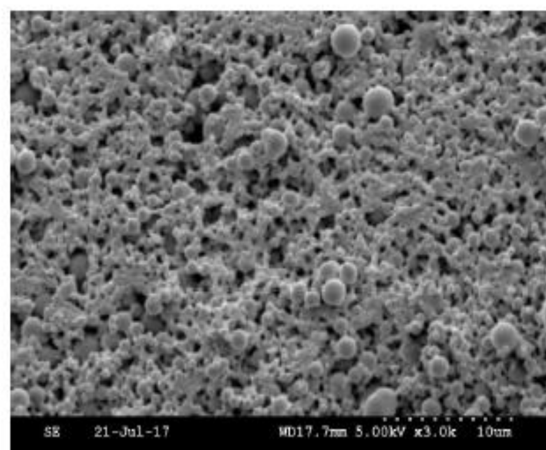

Fig 11: Showing SEM image for PLGA / caprolactone nanoparticles prepared by microfluidic technique

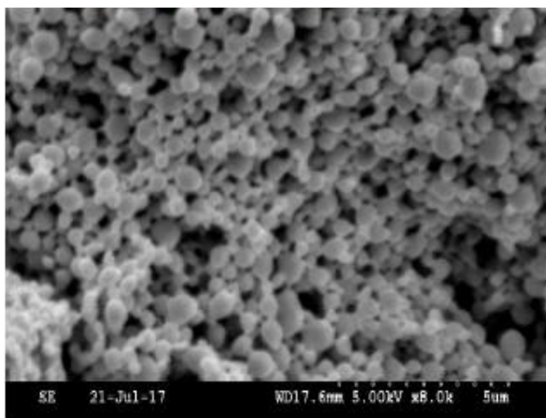

Fig 12: Showing SEM image for PLGA / caprolactone / poloxamer 188 nanoparticles prepared by doable emulsion

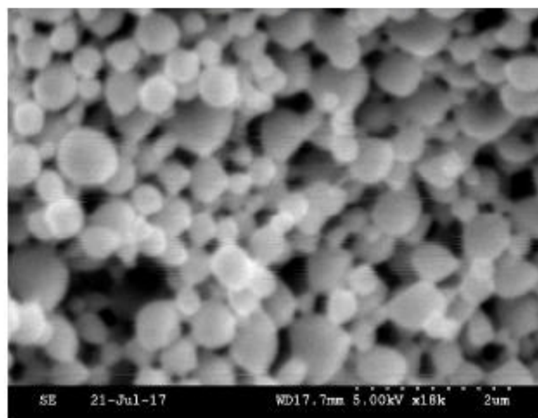

Fig 13: Showing SEM image for PLGA / caprolactone / poloxamer 188 nanoparticles prepared by microfluidic technique

3.4 Loading of insulin and encapsulation efficiency

For the nanoparticles prepared by microfluidic technique, PLGA with caprolactone copolymer nanoparticles and poloxamer 188 nanoparticles have the higher encapsulation value then PLGA and caprolactone copolymer nanoparticles and PLGA nanoparticles with the values 44.15 %, 43.14 % and 40.52 % (table 6, 7 & fig. 14).

Table 6: Showing results for release and encapsulation efficiency of insulin loaded nanoparticles manufactured double emulsion /solvent evaporation technique

Formulation ID	Abs 1	Abs 2	Abs 3	Abs avg.	conc. µ/mg	Encapsulation %	Polymer type	Encapsulation efficiency %
S1 DE	0.11	0.11	0.09	0.10	9.1	45.4	PLGA NPs	52.7 ± 15.8
S2 DE	0.15	0.16	0.15	0.15	13.7	68.5		
S3 DE	0.10	0.10	0.10	0.10	8.9	44.3		
S4 DE	0.09	0.10	0.10	0.10	8.5	42.6	PLGA / caprolactone copolymer NPs	42.4 ± 1.6
S5 DE	0.11	0.10	0.10	0.10	8.8	44.0		
S6 DE	0.08	0.10	0.09	0.09	8.1	40.5		
S7 DE	0.11	0.11	0.10	0.11	9.3	46.6	PLGA / caprolactone copolymer / poloxamer 188 NPs	45.1 ± 1.5
S8 DE	0.10	0.11	0.10	0.10	9.2	46.1		
S9 DE	0.10	0.09	0.09	0.10	8.5	42.6		

Table 7: Showing results for release co d encapsulation efficiency of insulin loaded nanoparticles manufactured by microfluidic / salting out technique

Formulation ID	Abs 1	Abs 2	Abs 3	Abs avg.	conc. µ/mg	Encapsulation %	Polymer type	Encapsulation efficiency %
S1 MF	0.09	0.09	0.09	0.09	8.0	40.1	PLGA NPs	40.5 ± 0.8
S2 MF	0.09	0.09	0.10	0.09	8.3	41.3		
S3 MF	0.09	0.08	0.10	0.09	8.0	40.2		
S4 MF	0.10	0.10	0.10	0.10	8.7	43.5	PLGA / caprolactone copolymer NPs	43.1 ± 1.7
S5 MF	0.09	0.10	0.09	0.09	8.2	41.1		
S6 MF	0.10	0.10	0.10	0.10	9.0	44.8		
S7 MF	0.09	0.11	0.11	0.10	9.2	45.8	PLGA / caprolactone copolymer / poloxamer 188 NPs	44.2 ± 1.6
S8 MF	0.10	0.10	0.10	0.10	8.9	44.5		
S9 MF	0.09	0.10	0.09	0.10	8.4	42.2		

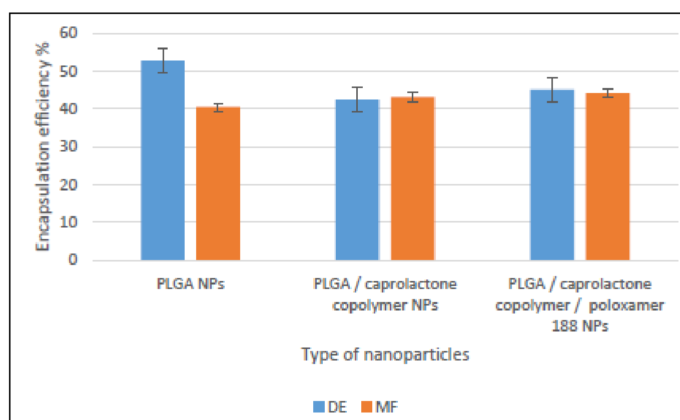

Fig 14: Graph showing the encapsulation efficiency of insulin loaded nanoparticles performed by both double emulsion / solvent evaporation and microfluidic / salting out techniques

3.5 *In vitro* release of insulin loaded nanoparticles

For the nanoparticles prepared by microfluidic technique as shown if figures (16), they are showing much lower initial burst release than nanoparticles prepared by double emulsion technique. However, PLGA nanoparticles are showing initial burst release with 12.29 % after 2 hours and full release of 57.64 % after seven days. furthermore, PLGA nanoparticles incorporated with caprolactone copolymer and PLGA nanoparticles with caprolactone copolymer and poloxamer 188 exhibit initial release of 10.12 % and 29.04 % after 2 hours and a full release of 41.04 % and 43.45 % after seven days respectively (fig 15).

Table 8: Showing *In vitro* cumulative release of insulin nanoparticles over seven days prepared by double emulsion solvent evaporation technic

Double emulsion / solvent evaporation technique						
	2 hrs	1 day	2 days	3 days	4 days	7 days
PLGA NPs	70.3%	80.8%	81.8%	80.8%	84.6%	93.3%
PLGA / caprolactone copolymer NPs	37.8%	47.2%	47.2%	49.3%	47.7%	48.5%
PLGA / caprolactone copolymer / poloxamer 188 NPS	36.2%	44.4%	44.4%	44.2%	42.0%	47.1%

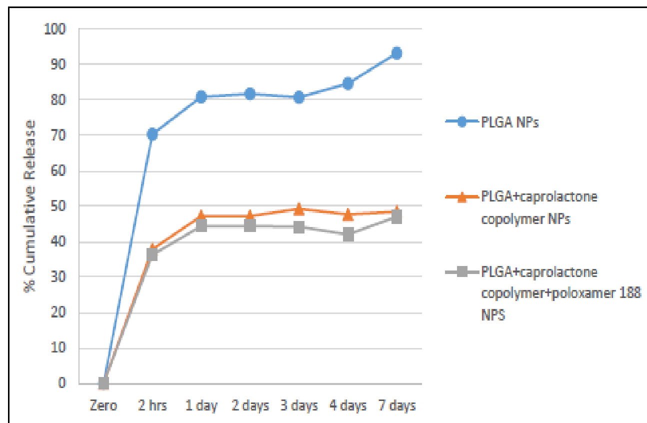

Fig 15: A graph showing *In vitro* cumulative release of insulin nanoparticles over seven days prepared by double emulsion / solvent evaporation technique

Table 9: Showing *In vitro* cumulative release of insulin nanoparticles over seven days prepared by microfluidic / salting out techniques

Micro fluidic / salting out technique						
	2 hrs	1 day	2 days	3 days	4 days	7 days
PLGA NPs	12.2 %	37.5 %	36.5 %	38.4 %	42.4 %	57.6 %
PLGA / caprolactone copolymer NPs	10.1 %	30.9 %	29.1 %	30.9 %	43.7 %	41.0 %
PLGA caprolactone copolymer / poloxamer 188 NPS	29.0 %	35.9 %	32.9 %	35.9 %	49.9 %	43.5 %

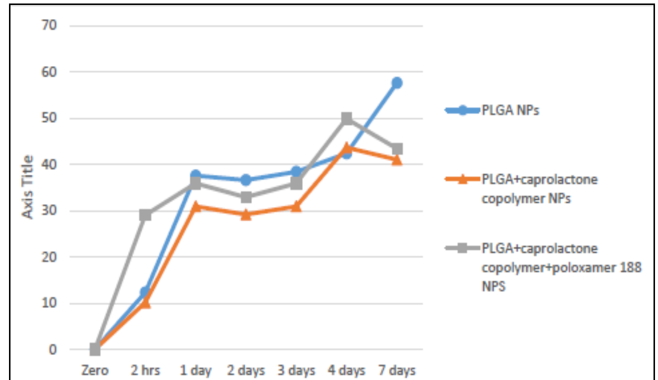

Fig 16: A graph showing *In vitro* cumulative release of insulin nanoparticles over seven days prepared by microfluidic /salting out technique

3.6 Stability and compatibility of insulin loaded nanoparticles

3.6.1 FTIR (Fourier transform infrared spectrophotometry)

Also for the nanoparticles prepared by PLGA with caprolactone copolymer for both double emulsion and microfluidic as shown in figure (19, 20) the nanoparticles chart was plotted against insulin, PLGA, PVA and caprolactone copolymer are showing matching between nanoparticles and PLGA and for the nanoparticles prepared by PLGA with caprolactone copolymer and poloxamer 188 for both double emulsions and microfluidic as shown in figure (21,22), the nanoparticles have the same peaks as PLGA polymer.

All the nanoparticles charts are matching with PLGA chart and other peaks disappeared and this indicates full encapsulation all the materials inside the nanoparticles.

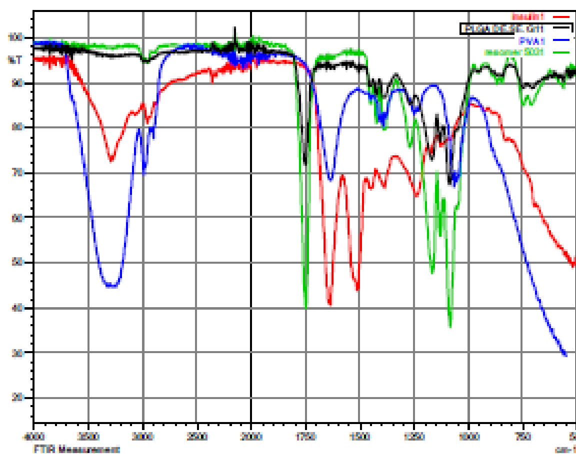

Fig 17: Showing FTIR chart for PLGA NPs and raw materials used in the preparation by double emulsion technique

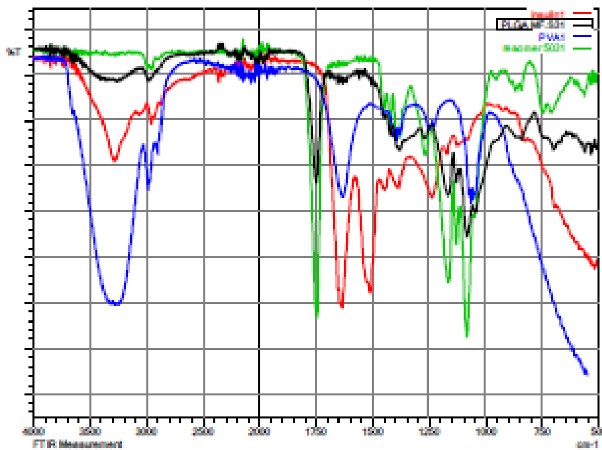

Fig 18: Showing FTIR chart for PLGA NPs and raw materials used in the preparation by microfluidic technique

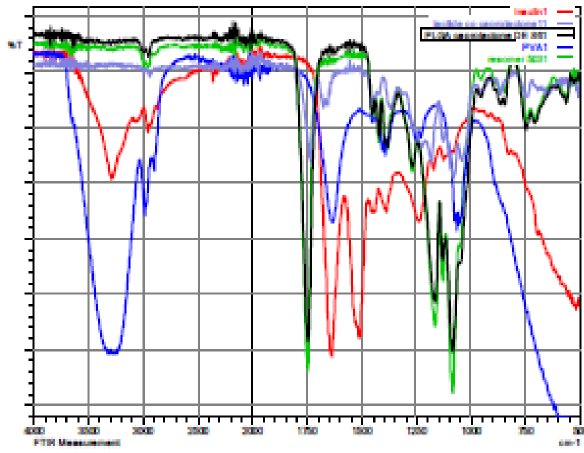

Fig 19: Showing FTIR chart for PLGA / caprolactone NPs and raw materials used in the preparation by double emulsion technique

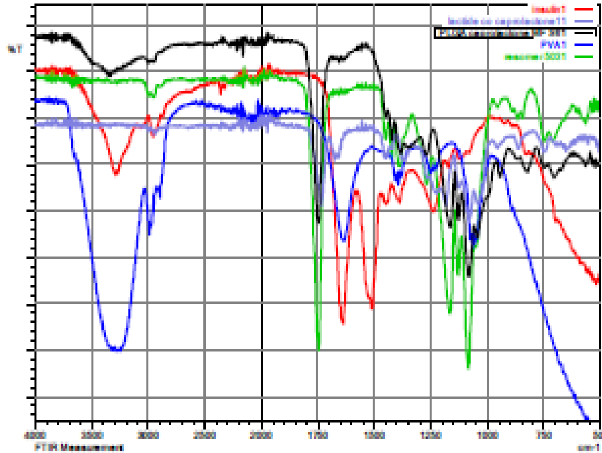

Fig 20: Showing FTIR chart for PLGA / caprolactone NPs and raw materials used in the preparation by microfluidic technique

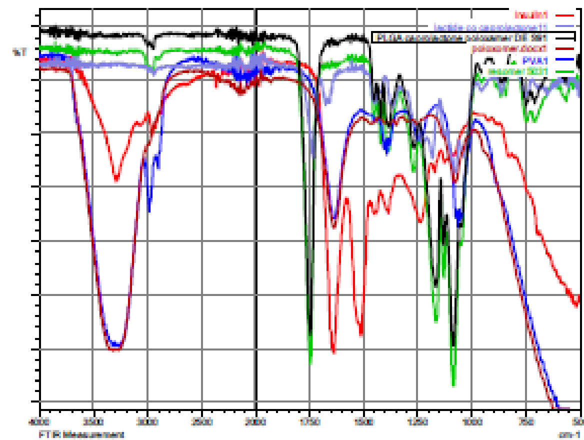

Fig 21: Showing FTIR chart for PLGA / caprolactone / poloxamer 188 NPs and raw materials used in the preparation by double emulsion technique

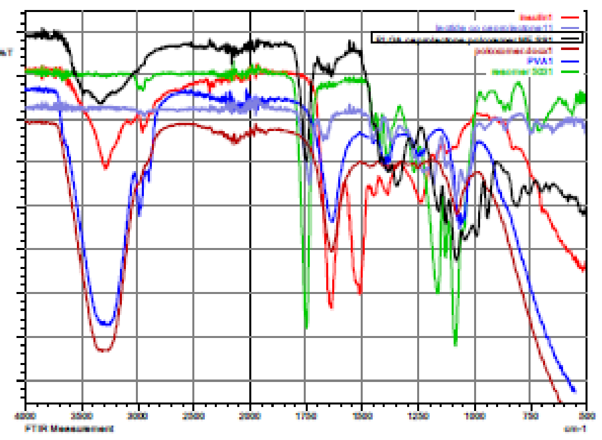

Fig 22: Showing FTIR chart for PLGA / caprolactone / poloxamer 188 NPs and raw materials used in the preparation by microfluidic technique

3.6.2 DSC (Differential scanning calorimeter)

PLGA nanoparticles prepared by double emulsion and microfluidic techniques as shown in figure (23 and 24) are showing a transition temperature (Tg) at about 49 °C the same as PLGA polymer. Incorporation of caprolactone copolymer to the nanoparticles as shown in figure (25 and 26) didn't affect the transition temperature (Tg) which is about 49 °C the same as PLGA. Also incorporation of poloxamer 188 has no effect on the transition temperature for the nanoparticles as shown in figure (27 and 28) which is 49 °C.

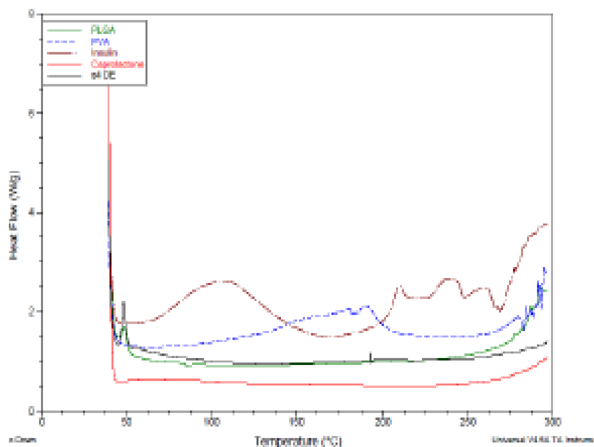

Fig 23: Showing DSC chart for PLGA NPs and raw materials used in the preparation by double emulsion technique

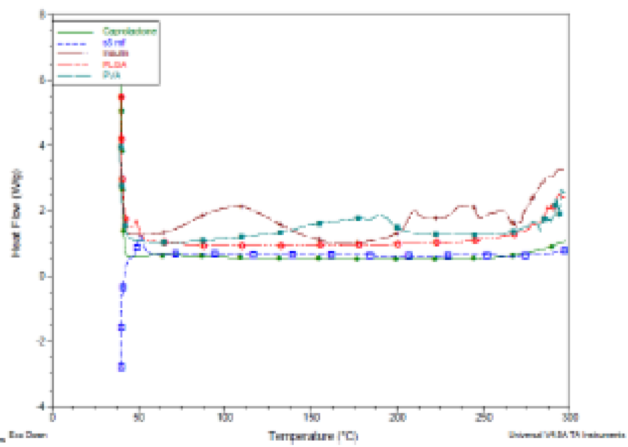

Fig 24: Showing DSC chart for PLGA NPs and raw materials used in the preparation by microfluidic technique

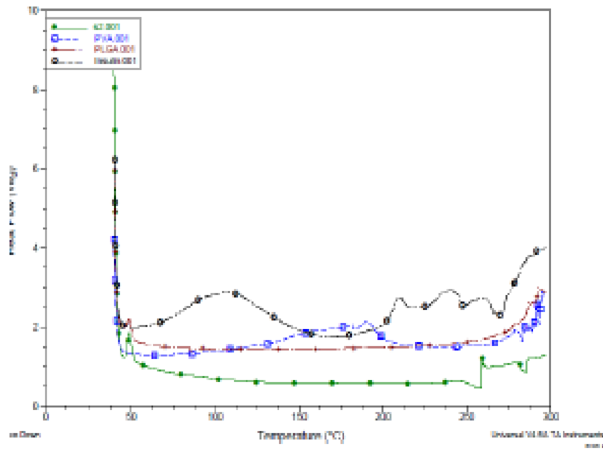

Fig 25: Showing DSC chart for PLGA / caprolactone NPs and raw materials used in the preparation by double emulsion technique

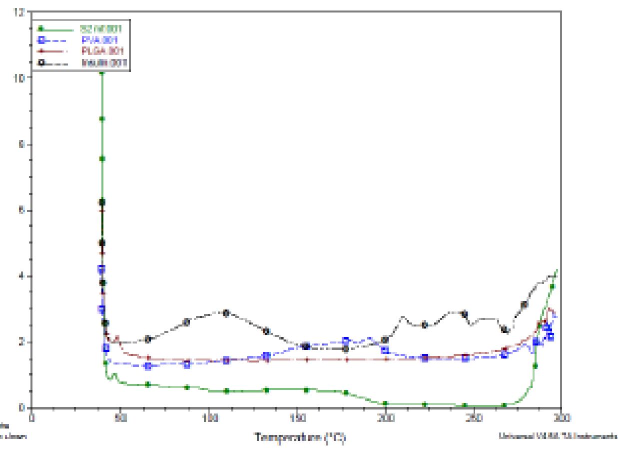

Fig 26: Showing DSC chart for PLGA / caprolactone NPs and raw materials used in the preparation by microfluidic technique

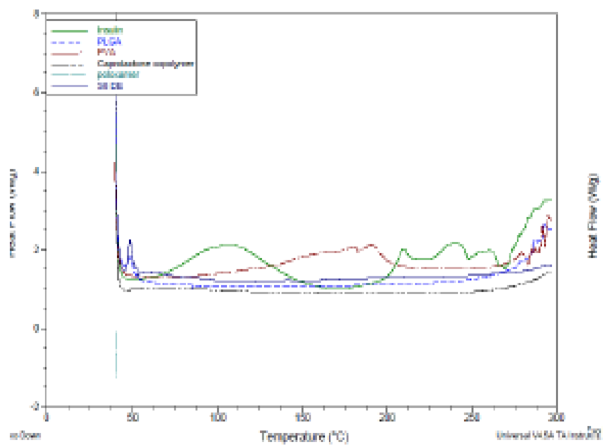

Fig 27: Showing DSC chart for PLGA / caprolactone / poloxamer 188 NPs and raw materials used in the preparation by double emulsion technique

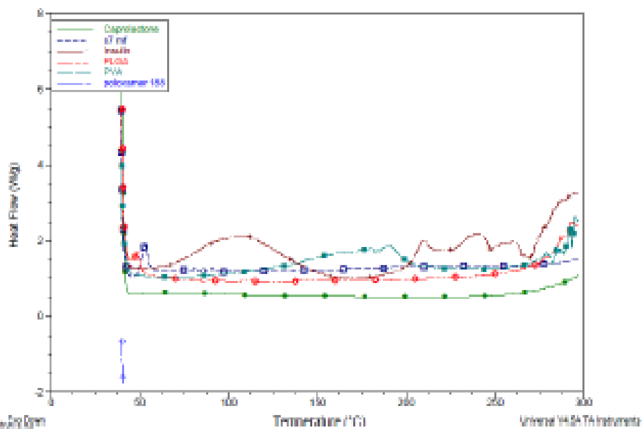

Fig 28: Showing DSC chart for PLGA / caprolactone / poloxamer 188 NPs and raw materials used in the preparation by microfluidic technique

4. Discussion

4.1 Method of preparation

The homogenization steps of the emulsion technique are associated with random shear distribution which leads to high variation in the droplets size in each step of the preparation. Also, the high homogenization force might lead to the escape of inner droplets from the outer droplets that might leads to low encapsulation efficiency. Also,

The double emulsion consists of many steps in the preparation which consumes a lot of time. Solvent evaporation was applied next to remove the acetonitrile which is a toxic volatile organic solvent by stirring the emulsion for 24 hours at atmospheric temperature or lower which is also a time-consuming method [10].

The second technique used is microfluidic/salting out technique. Recently, microfluidic technique is being used a lot in many researches for the preparation of micro and nanoparticles which works by exploiting controlled mixing of streams in micro-sized channels [11]. Microfluidic fluidic technique has a superior control over the size and dispersity of the particles over the double emulsion technique. Furthermore, has the advantage of being easy to use and the parameters like flow rate and flow rate ratio can be easily adjusted using its software Also, it doesn't consume time as the preparation of each sample took only a few minutes. For the removal of acetonitrile which is a toxic volatile organic solved, salting out method was used by sodium borate which consumes much less time than the solvent evaporation [7].

4.2 Polymers used in the preparations

PLGA Resomer® RG 503 H is a type of Poly (DL-lactide-co-caprolactone) polymers which considered one of the most attractive polymers used now. It is a non-toxic, biocompatible and biodegradable polymer. It is a poly ester consists of poly lactic and poly glycolic acid. Lactic acid is less hydrophilic than glycolide so the ratio between them controls the hydrophilicity of the polymer. Also, the molecular weight can be controlled by changing the length of the polymer chain. PLGA degradation is performed by the hydrolysis of the ester bond between lactic and glycolide acid and it exhibits bulk degradation more than surface degradation [6]. PLGA is compatible with most of the drugs and considered has a perfect host especially for hydrophilic drugs. Resomer® RG 503 H has a ratio 50:50 between lactic and glycolic acid with moderate hydrophobicity [12].

Poly (DL-lactide-co-caprolactone) shares a lot of properties with PLGA as it is a hydrophilic, biocompatible and biodegradable polymer. It is prepared by the esterification of lactide and caprolactone the ratio between them controls the hydrophilicity of the drug. The type used in this research has a ratio of 85:15 between lactide and caprolactone with high

molecular weight. The degradation occurs by hydrolysis of the ester bond to smaller molecules and bulk erosion is more susceptible than surface erosion [13].

Poloxamer 188 is a nonionic surfactant which has a hydrophobic part of polyoxypropylene and a hydrophilic part of polyoxyethylene. poloxamer is a surface active agent so by incorporation poloxamer in the preparation of the nanoparticles it improves the dispersion of the particles by acting as a co-emulsifier and gives a smaller size of nanoparticles. also, the hydrophilicity of the polymer improves the porosity which improve the release behaviour of the nanoparticles.

4.3 Size

Size is very critical for the loading of the drug as by decreasing the size the loading capacity is increased and it is very critical for the release patterns as well. The size of the particles determines the release of the drug as by decreasing the size

Two different techniques have been used in the preparation double emulsion / solvent evaporation and microfluidic / salting out which have a big influence on the size of the nanoparticles. However, nanoparticles prepared by double emulsion technique have bigger size than nanoparticles prepared by microfluidic technique with a larger distribution and the reason for that multi emulsions techniques like the double emulsion includes multi sequential emulsification steps which are associated with high random shear distribution which leads to broad size distribution of the nanoparticles in each emulsification step. On the other hand, microfluidic is a promising new technique providing the capability of nanoparticles preparation just in one step. Furthermore, particle size and its distribution can be controlled efficiently providing smaller nanoparticles with low PDI.

The polymers used have a non-significant influence on the size of the nanoparticles. Three different polymers have been used in the formulations PLGA Resomer® RG 503 H, Poly (D, L-lactide-co-caprolactone) and Poloxamer 188. The addition of these polymers has been performed by physical mixing. In addition, all these polymers are hydrophobic and have a close molecular weight so the size of the nanoparticles is slightly affected. However, PLGA nanoparticles have the biggest size for both double emulsion and microfluidic. Incorporation of 5 % of caprolactone copolymer by physical mixing to the nanoparticles slightly decreased the particle size average for the double emulsion technique around 5 nm and around 7 nm for nanoparticles prepared by microfluidic. Addition of 5 % poloxamer 188 also slightly decreased the average size of the nanoparticles by 10 nm for the double emulsion and 4 nm for the microfluidic.

4.4 Zeta potential

Results of Zeta potential for the insulin nanoparticles are showing that the preparation technique has the biggest influence on the zeta potential as the nanoparticles prepared by microfluidic technique have more negative charge than those prepared by double emulsion technique which is mostly related to the difference in the organic solvent used in each technique as acetonitrile was used as organic solvent for microfluidic technique and dichloromethane was used as an organic solvent for the double emulsion technique.

For the microfluidic technique comes the PLGA with caprolactone copolymer and poloxamer 188 with the highest average then PLGA nanoparticles then PLGA with caprolactone copolymer nanoparticles. But, the difference in zeta potential between the nanoparticles in each method of preparation is very close and non-significant.

4.5 Morphological analysis and imaging

The nanoparticles prepared by double emulsion technique are showing more variation in size and higher poly dispersity than the particles prepared by microfluidic technique as microfluidic technique can provide a homogenous distribution of nanoparticles. Incorporation of caprolactone copolymer and poloxamer 188 to the nanoparticles have no effect on the morphological characterization of the nanoparticles [14].

4.6 Loading and encapsulation efficiency of the insulin loaded nanoparticles

For the nanoparticles prepared by double emulsion technique, PLGA nanoparticles comes with the highest encapsulation efficiency with average of 52.74 % which was not expected to be the highest as it was expected for PLGA with caprolactone and poloxamer to be the highest because as poloxamer can help in nanoparticles formation in the primary emulsion phase and it comes with encapsulation efficiency of 45.11 % then last comes PLGA with caprolactone nanoparticles with average EE % of 42.38 %.

But for the nanoparticles prepared by microfluidic technique, as expected PLGA with caprolactone and poloxamer 188 nanoparticles have the highest encapsulation efficiency with average of 44.15 %, then comes PLGA with caprolactone nanoparticles and PLGA nanoparticles with average encapsulation efficiency of 43.14 % & 40.52 % respectively.

4.7 *In vitro* release of insulin loaded nanoparticles.

Nanoparticles prepared by microfluidic technique have been showing improved pattern of release than those prepared by double emulsion technique especially after the addition of poloxamer 188 but still the full release needs some improvement as high amount of insulin still entrapped inside the nanoparticles. This might be improved by addition of hydrophilic polymer which can facilitate the diffusion of the drug and decrease the entrapped amount of the drug inside the nanoparticles.

4.8 FTIR (Fourier transform infrared spectrophotometry)

The spectrum of PLGA nanoparticles has been plotted against insulin, PVA and PLGA. For the PLGA with caprolactone copolymer nanoparticles the spectrum has been plotted against insulin, PVA, PLGA and caprolactone copolymer. For the PLGA with caprolactone copolymer and poloxamer 188 nanoparticles the spectrum has been plotted against insulin, PVA, PLGA, Caprolactone copolymer and poloxamer 188.

All the nanoparticles are showing matching between their spectrum and PLGA spectrum which is showing a stretching peak of (C-O-C) group at 1088 cm-1, and a methyl stretching (C-H) between 1400 and 1500 cm-1, main peak of stretching carbonyl group (C-O-O) between 1700 and 1800 cm-1 and stretching (CH, CH2, CH3) groups between 2800 and 3000 cm-1 [15].

From the previous data we can confirm that all the ingredients of the nanoparticles are entrapped inside with no

chemical interaction between them which confirm the compatibility between the ingredients.

4.9 DSC (differential scanning calorimetry)

All the nanoparticles are showing the same peak as PLGA and other peaks of the ingredients disappeared in the nanoparticles thermograms which confirm the stability and compatibility of the ingredients of the nanoparticles with no thermal interactions.

5. Conclusions

Incorporation of Poly (DL-lactide-co-caprolactone) has shown decrease in the size average and it was decreased more after the addition of poloxamer 188 which acts as a co-emulsifier in the formulations prepared by the both techniques. Also, the microfluidic technique has shown improvement and decrease in the average size than the double emulsion technique with less variation in the size dispersion as the microfluidic technique has much better control on the size and dispersity of the nanoparticles over the double emulsion technique.

6. References

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