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Development of Amoxicillin loaded microspheres for anti-*Helicobacter pylori* infection using Ionic Gelation method

Fashli Razak[1], Noreen Morris[1], Alan Murphy[1], James Kennedy[1]

[1] Material Research Institute, Athlone Institute of Technology, Republic of Ireland

Abstract

The aim of this project was to develop microspheres loaded with Amoxicillin for anti-*Helicobacter pylori* infection. Amoxicillin loaded microspheres were developed using blends of natural polymers such as Alginate and Chitosan using an Ionotropic gelation method. In this study, the physiochemical properties such as particle size, surface morphology, production yield, drug loading, entrapment efficiency, swelling index, and *in vitro* drug release mechanism were analysed. From this study, it was found out that AMX06 had the highest particle size (653µm) and drug loading (68%) while AMX01 had the highest production yield and entrapment efficiency (80%). All formulations demonstrated a burst drug release profile and AMX04 provided the best drug release profile (89%) after 6 hours. The FTIR analysis proved that a reaction had been established between the materials with the presence of a peak at 1772 cm^{-1} indicating the presence of a β-lactam ring of Amoxicillin and peak at 1580 cm^{-1} indicates the N-H bending of Chitosan amino group.

Keywords: *Amoxicillin, Chitosan, Alginate, Microsphere, Ionic gelation method.*

1. Introduction

Helicobacter pylori (H. pylori) have been reported to affect 50% of the world population, making it the most common bacterial infection known to the medical community (Siddalingam, 2014). This bacteria was first discovered by two Australian scientists, Robin Warren and Barry Marshall in 1982 (Warren & Marshall, 1983). The ability of this bacterium to survive in low pH environments makes it favourable to be found in the antrum of the stomach as shown in Figure 1 (Zhao et al., 2014). *H. pylori* has been widely known as the root cause of acute and chronic gastritis and is a major causative factor leading to several ulcer diseases, inflammation and cancer as presented in Figure 2 (Sachs & Scott, 2012).

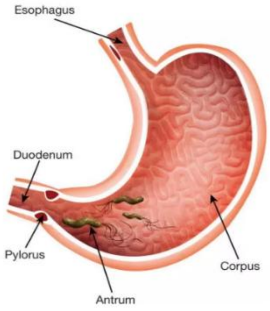

Figure 1: Illustration diagram of *H. pylori* reside in the antrum of the stomach (Brock, 2012)

The treatment goal for *H. pylori* infection is the complete eradication of the microorganism from the gastrointestinal tract. The success of the treatment can be described as when there are no signs of *H. pylori* presence after 28 days of the treatment therapy (Harris, 1998). The recommended first-line treatment for *H. pylori* infection is a combination therapy consisting of one Proton Pump Inhibitor, Omeprazole and two antibacterial agents; Amoxicillin and Clarithromycin (Zhao et al., 2014).

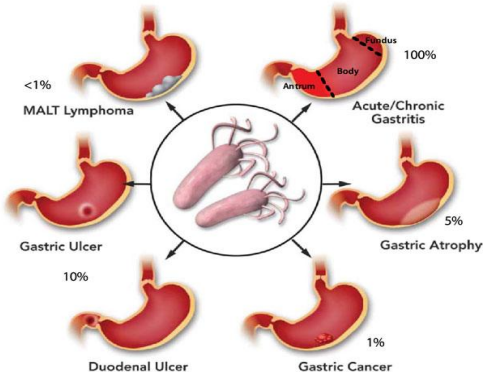

Figure 2: Consequences of *H. plyori* infection

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There are a lot of limitations to the current treatment and it is very important to ensure a substantial concentration of Amoxicillin is provided for the treatment of *H. Pylori* infection. Conventional Amoxicillin tablets are very sensitive to the acidic nature (pH 1-2) of the human stomach (Lozniewski et al., 1999). Thus, it is important to shorten the Amoxicillin residence time in the stomach. If the bacteria is not fully eradicated, it will increase the chances of re-colonization of *H. pylori* in the stomach epithelial lining (Angadi, Manjeshwar, & Aminabhavi, 2012).

As presented in Figure 3, Amoxicillin originates from an antibiotic group known as penicillin, a β-lactam antibiotic. It is an acid resistant drug and has been proven to fight off gram positive and some gram negative bacteria in humans and animals which include *H. pylori* infection (Rao, Kaur, & Nanda, 2011). Common side effects of Amoxicillin are nausea, vomiting, and diarrhoea. Amoxicillin mode of action on bacteria is to binding to a serine unit in the enzyme responsible for cell wall synthesis thus disrupting this synthesis (Weber, Tolkoff-Rubin, & Rubin, 1984).

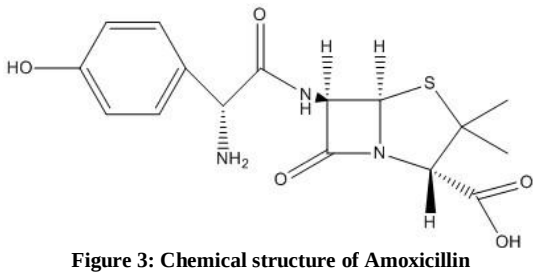

Figure 3: Chemical structure of Amoxicillin

The matrix network structure produced by polymers can help the drug to be released in a controlled manner. Historically, Smith and Kline and French introduced the controlled released formulation known as Spansule® in 1952. The controlled release capsules encapsulate hundreds of micro pellets and are released in 12-hour intervals (Park, 2015). The main advantage of controlled drug delivery systems is that the drug can be released over a sustained period at a nearly constant rate. This leads to improved medication adherence for the patient as the drug intake frequency is reduced.

In this work, Amoxicillin was added into Chitosan-Alginate microspheres prepared using the Ionic Gelation method. The physicochemical properties such as particle size, surface morphology, production yield, drug loading, entrapment efficiency, swelling index and *in vitro* drug release mechanism were analysed in an effort to better understanding of the stability of the drug release system.

2. Methods

2.1 Materials and Instrumentation

Amoxicillin trihydrate (CAS no. 61336-70-7; MW 365.40; assays >98.0%) was purchased from Tokyo Chemical Industry (Belgium). Chitosan medium molecular weight (75-85% deacetylation; viscosity 200-800cps; product no. 448877; CAS no.9012-76-4), Sodium Alginate (viscosity 5.0-40.0 cps; product no. W201502, CAS no. 9005-38-3), Dioctyl Sodium sulfosuccinate (DOSS; assay ≥97%; MW 444.56; EC no. 209-406-4), Glacial acetic acid (Product no. A6283, MW 60.05; CAS no. 64-19-7; Reagent ≥99%), Sodium hydroxide (ACS reagent ≥ 97%; Product no. 221465; CAS no. 1310-73-2), Calcium Chloride (MW 111.0; CAS no. 10043-52-4; assay ≥93%) were all purchased from Sigma Aldrich (Wicklow, Ireland).

2.2 Preparation of Amoxicillin loaded microspheres

The production of Amoxicillin loaded microspheres was carried out using the Ionic gelation method. Sodium Alginate powder (2-4 % w/v) was dissolved in a 20 mL beaker containing distilled water and using vigorous stirring (500 rpm). When the Alginate powder had dissolved, Amoxicillin (2-6 % w/v) powder was suspended in the solution and allowed to homogenize for two hours. Then the mixture was sprayed using a syringe into the gelation medium containing Chitosan (0.25 %w/v) and Calcium Chloride ($CaCl_2$) (4% and 6 % w/v). The gelation medium solution pH was adjusted to pH 5 using Sodium Hydroxide (1M).

2.3 Formulation

Six formulations from the optimization studies were chosen based on the surface morphology from the previous experiment (Razak, Chyzna, Morris, Murphy, & Kennedy, 2017). The Amoxicillin loaded microsphere samples were labelled AMX01 to 06.

Table 1: Various formulation

AMX	Amox	SA	CHS	$CaCl_2$	D:P ratio
01	4	4	0.25	4	1:1
02	2	4	0.25	4	1:2
03	2	2	0.25	4	1:1
04	4	4	0.25	6	1:1
05	2	4	0.25	6	1:2
06	6	3	0.25	6	2:1

Amox: Amoxicillin; SA: Sodium Alginate; CHS: Chitosan, $CaCl_s$: Calcium Chloride; D:P: Drug: Polymer. All material weight in (% w.v).

2.4 Particle size

The particle size determination was determined by light microscopy (Leica Wild M3Z Stereomicroscope) equipped with a video camera (TK-C1381 JVC) and computer software (Buehler Omnimet). 50 randomly selected microspheres were analysed from each formulation and the mean diameter was calculated using the Edmondson equation (Equation 1):

$$D\ mean = \frac{\Sigma\ nd}{\Sigma\ n}$$

n= number of microspheres
d= mean size range

(Equation 1)

2.5 Surface morphology

The surface morphology of the microspheres was examined using Tescan MIRA XMU SEM and the surface elemental composition was analysed with Energy Dispersive X-ray Spectroscopy, EDX using a Silicon Drift Detector (SDD) X MAX. Preparation of the samples for SEM microscope examination included the microspheres being placed on an aluminium stub coated with double sided adhesive tape. The samples were coated with gold alloy in an argon atmosphere using the Baltec SC 005 instrument for 110 seconds at 0.1 mBar in order to provide electrical conductivity on the surface of the samples.

2.6 Production yield

The production yield was calculated in percentage to identify the microspheres recovery efficiency. The microspheres production yield was identified by calculating the practical yield which is the initial weight of dispersed medium (Sodium Alginate and Amoxicillin) and theoretical yield which is the last weight of microspheres produced as shown in equation 2 (Agrawal, Wakte, & Shelke, 2017). This experiment was carried out in triplicate.

$$Production\ yield\ (\%) = \frac{Practical\ yield}{Theoretical\ yield} \times 100\%$$

(Equation 2)

2.7 Calibration curve

A standard stock solution of Amoxicillin was prepared by dissolving 100 mg of Amoxicillin in 100 ml of pH 1.2 buffer to produce a concentration of 1 mg/ml. Then, the sample was scanned using a UV-Vis Spectrophotometer in the range of 190-400 nm using the pH 1.2 buffer as a blank. The stock solution was diluted to obtain a concentration range of 0.5 mg/ml to 0.0625 mg/ml. Absorbance of each of the solutions was measured at 271 nm using Shimadzu UV 1280 UV-VIS Spectrophotometer. The experiment was performed in triplicate and an average absorbance was calculated; the calibration curve was obtained by plotting average absorbance versus concentration data. The linear plot value obtained is y= 2.4533 x + 0.0059 and R^2 value of 0.9998.

2.8 Drug loading and Entrapment Efficiency

50 mg of Amoxicillin loaded microspheres were accurately weighed and crushed using a mortar and pestle. The powdered microspheres produced were then suspended in 100 ml of pH 1.2 buffer. After 24 hours, the solution was filtered using 0.45 µm membrane filter. Of this, 2 ml of the filtrate was taken and diluted to 10 ml. The absorbance was measured at 271 nm and analysed using the calibration curve (Section 2.7). The values were then multiplied by 10 ml to obtain the practical value. Drug Loading and Entrapment Efficiency were calculated using the formula in Equation 3 and Equation 4, respectively.

$$Drug\ loading\ (\%) = \frac{Weight\ of\ drug\ in\ microspheres}{Weight\ of\ microspheres}\ x\ 100\%$$

(Equation 3)

$$Entrapment\ efficiency(\%) = \frac{Weight\ of\ total\ drug - weight\ of\ free\ drug}{Weight\ of\ total\ drug} \times 100\%$$

(Equation 4)

2.9 Fourier Transform Infrared Spectroscopy

The functional groups of the Amoxicillin loaded microspheres were studied using a Perkin Elmer Spectrum One FTIR Spectrometer. The sample was finely grounded using a mortar and pestle and placed on the ATR crystal. Data was collected in the spectral range of 4000-650 cm^{-1} at 21°C, utilising a 4 scan per sample cycle and fixed universal compression force of 80 N. Subsequent analysis was carried out using Spectrum software, Perkin Elmer.

2.10 Swelling study

The swelling index of microspheres was determined by immersing 50 mg of Amoxicillin loaded microspheres in buffer pH 1.2 and the weight of the microspheres was determined at pre-determined time intervals. The excess surface adhered liquid drops were removed by blotting with filter paper and

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microspheres were weighed using a balance. The swelling index was calculated from the difference between the initial weight of the microspheres and the weight at the time of determination using Equation 5.

$$Swelling\ index = \frac{(Wt - Wo)}{(Wo)}\ x\ 100\%$$

Wt= Weight at pre-determined time point
Wo= Weight at initial

(Equation 5)

2.11 *In vitro* Drug Release from microspheres

The *in vitro* Amoxicillin release from microspheres was investigated using the USP II paddle apparatus method containing 900 mL of freshly prepared buffer of pH 1.2. Each formulation of Amoxicillin loaded microspheres was accurately weighed (50 mg) and allowed to sink to the bottom of the dissolution vessels containing the buffer medium. The buffer was made up from Potassium Chloride (KCl) and Hydrochloric Acid (HCl) and was maintained at 37 ± 0.5 °C and the paddle rotated at 50 rpm.

Each dissolution test was conducted in triplicate. 3 mL was withdrawn from the sample every 15 minutes for the first hour and continuously on an hourly basis for the next 5 hours. The sample withdrawn was filtered through a membrane filter and was replaced with the same amount using a fresh buffer solution. The absorbance of the sample withdrawn was then measured with a UV spectrophotometer, Shimadzu UV-1280 UV-Vis Spectrophotometer.

The absorbance of the blank microspheres was measured and subtracted from the absorbance of the sample to remove any interference from the polymers used.

3. Results and Discussion

3.1 Results

3.1.1 Amoxicillin loaded microspheres

The Amoxicillin loaded microspheres were successfully prepared using Ionic Gelation method. The result of the six formulations for Amoxicillin loaded microspheres such as particle size, surface morphology, calibration curve, production yield, drug loading, entrapment efficiency, FTIR, swelling index results and *in vitro* drug release mechanism were analysed. Table 2 presents the results of:

i. Particle Size
ii. Production Yield
iii. Drug Loading
iv. Entrapment Efficiency

Table 2: Particle size, Production yield, Drug loading, and Entrapment efficiency of formulated Amoxicillin loaded microspheres.

AMX	PS (µm)	PY (%)	DL (%)	EE (%)
01	487 ± 0.05	86.33 ± 1.15	46.22 ± 0.04	80.11 ± 0.07
02	409 ± 0.05	63.67 ± 13.28	14.64 ± 0.03	29.17 ± 0.07
03	412 ± 0.05	78.33 ± 8.08	30.23 ± 0.06	45.73 ± 0.10
04	540 ± 0.06	76.67 ± 6.35	34.79 ± 0.06	58.47 ± 0.10
05	355 ± 0.04	64.67 ± 7.77	15.95 ± 0.03	18.78 ± 0.04
06	653 ± 0.09	68.5 ± 21.92	68.11 ± 0.10	70.07 ± 0.11

PS: Particle Size; PY: Production Yield; DL: Drug Loading; EE: Entrapment Efficiency.

3.1.2 Particle Size

AMX06 showed the largest particle size with a measurement of 653 ± 0.09 µm, whereas AMX05 produced the smallest particle size (355 ± 0.04 µm). To determine the effect of Amoxicillin concentration on the formulation, the formulation AMX01 was compared with AMX02 while AMX04 with AMX05. Both formulations have the same concentration of polymer used however they are different in the Amoxicillin concentration. AMX01 and AMX04 both have 4% w/v Amoxicillin while AMX02 and AMX05 have 2% w/v. From the investigation, it indicates that there is a significant effect (*t*-test, $p < 0.05$) on the particle size when the concentration was decreased from 4% w/v to 2% w/v.

The effect of Sodium Alginate concentration on Particle Size and Surface Morphology was determined by comparing AMX02 and AMX03 with a concentration of 4% w/v and 2% w/v respectively. It shows that there is no significant difference produced when comparing these 2 parameters ($p > 0.05$). It can be noted that there is a significant difference produced when comparing the concentration of $CaCl_2$ in AMX01 and AMX04 (*t*-test, $p < 0.05$).

Three different ratios of Amoxicillin to Sodium Alginate (1:1, 1:2 and 2:1) were selected to determine the effect of the drug/polymer ratio on the

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formation of microspheres. The formulations with a 2:1 ratio of Amoxicillin to Sodium Alginate provide the biggest particle size when compared to the other two formulations ($p < 0.05$). On the other hand, the smallest particle size is produced with a 1:2 drug/polymer ratio (AMX05). Figure 4 provides the result of the particle size analysis.

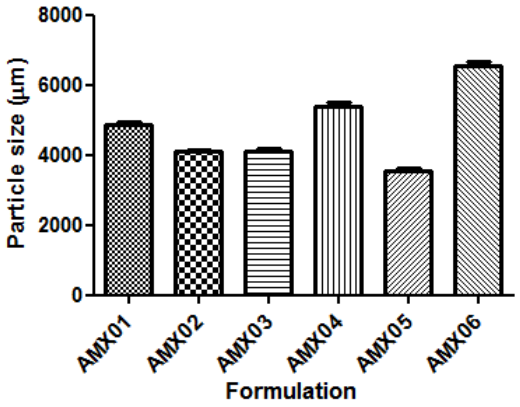

Figure 4: Bar Chart comparing the particle size of Amoxicillin loaded microspheres with different formulations.

3.1.3 Surface Morphology

The SEM images of the composite beads and their surface were taken. Four formulations were investigated for this purpose, namely: AMX01, AMX04, AMX05 and AMX06 as shown in Figure 5.

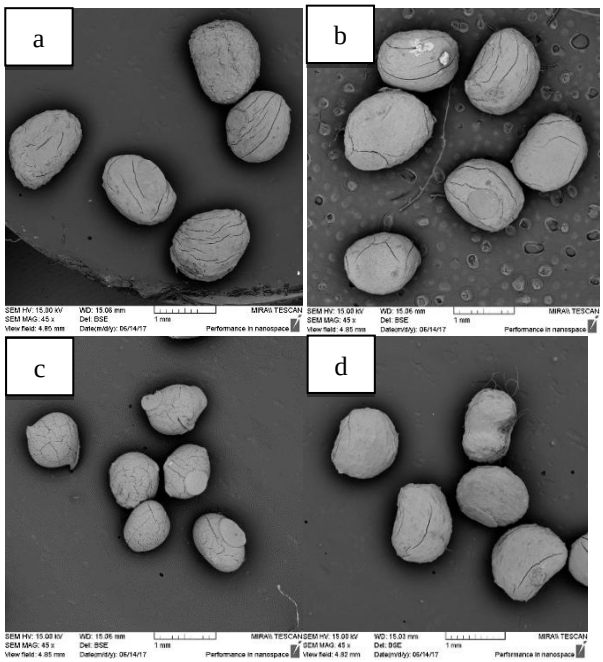

Figure 5: SEM images of a) AMX01, b) AMX04, c) AMX05 and d) AMX06. SEM magnification of image 1 is 55x and 2, 3 and 4 are 45x.

SEM imaging was performed in order to obtain the shape and surface structure of the Amoxicillin loaded microspheres. Figure 5 demonstrates that all formulations produced an almost spherical shape. It was seen under light microscopy that in a wet state the microsphere had a perfectly round shape. However, the perfect spherical shape was then distorted after the drying process. The distorted spherical structure after drying process was caused by a dense layer with a loose core produced as a result of the heterogeneous gelation mechanism that is commonly found in Calcium-Alginate layered by Chitosan beads (Angadi et al., 2012).

The presence of the crack line on the surface of the microspheres can be attributed to the rapid drying process and over exposure to heat (37 °C) in the oven. A higher Amoxicillin concentration (AMX06) produced a larger particle size as it increased the viscosity of the dispersed phase which can be seen when comparing AMX05 (Figure 5c) and AMX06 (Figure 5d) respectively. A high concentration of Amoxicillin will also have a denser layer structure as it spreads through the beads (Sahasathian, Praphairaksit, & Muangsin, 2010).

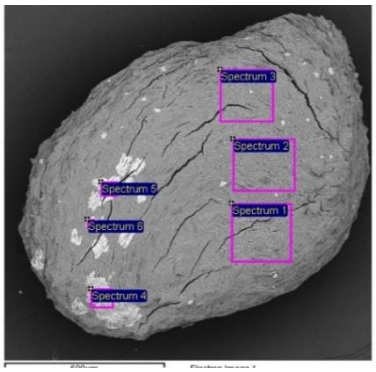

Figure 6: An SEM image of AMX01 sample used for EDX analysis showing the 6 locations chosen on the bead (Spectrum 1 to 6)

Table 3 presented the EDX analysis of surface composition results for AMX01 as shown in Figure 6. The highest composition observed on the surface of the bead (Spectrum 1) is Oxygen followed by Carbon being the second highest except for AMX06. Alginic acid is believed to be the source of the Oxygen. The same result was observed in other samples.

Table 3: EDX results for 6 different locations on AMX01 bead.

Spe	C	O	Na	Cl	Ca
1	27.71	42.31	2.01	9.14	11.55
2	-	44.89	3.37	15.66	22.20
3	28.91	44.35	2.20	7.79	10.31

4	37.45	3.68	8.34	34.44	2.78
5	14.44	6.75	24.34	41.84	4.27
6	19.57	2.47	23.44	44.83	1.96

Spec: Spectrum; C: Carbon, O: Oxygen, Na: Sodium; Cl: Chloride; Ca: Calcium. All the values are in %.

Upon the polyelectrolyte interaction between the dispersed phase and the gelation medium, the substitution reaction between Sodium Alginate and $CaCl_2$ leads to the formation of Sodium Chloride precipitate (white precipitate on the surface of the microspheres that is represented by Spectrum 4, 5 and 6). This salt precipitation can be removed by rinsing the bead repetitively with distilled water before drying it in the oven.

3.1.4 Production yield

The production yields for all six formulations are shown in Table 2 (column 2) and Figure 7. AMX01 provides the highest production yield at roughly 86% production yield. Production yield results for all 6 formulations of microspheres varied from ~63% up to 86%. The effect of $CaCl_2$ concentration on the yields was determined by comparing AMX01 (86.33 ± 1.15%) and AMX04 (76.67 ± 6.35%) which contains 4% w/v and 6% w/v of $CaCl_2$, respectively. When comparing these 2 formulations, there were no significant differences reported regarding the production yield (unpaired *t*-test, $p > 0.05$).

Different Amoxicillin ratios to Sodium Alginate (1:1, 1:2 and 2:1) represented by AMX01, AMX04 and AMX06 were selected to determine the effect on the production yield. It was discovered that formulations with a drug/polymer ratio of 1:1 (AMX01, AMX03 and AMX04) provided the best production yields.

There was no increase in the production yield observed when the concentration of Amoxicillin was increased to 6% w/v (ANOVA, $p > 0.05$). These experiments were carried out in triplicate to produce an average and accurate result.

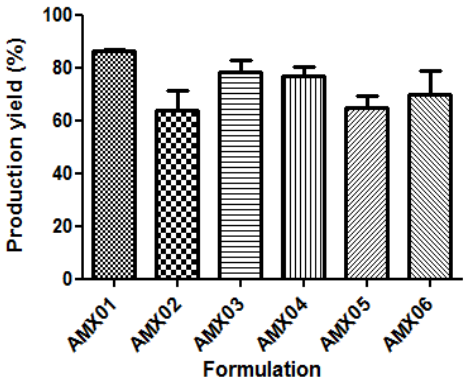

Figure 7: Comparison of Production Yields for Amoxicillin loaded microspheres.

3.1.5 Drug loading

Based on the result obtained (Table 2, column 3), AMX06 demonstrates the highest drug loading, 68.11 ± 0.10%, whereas, the lowest drug loading was observed with AMX02, 14.64 ± 0.03%. Three different Amoxicillin to Sodium Alginate ratios were selected: 1:1, 1:2 and 2:1 to determine the effect(s) of Amoxicillin concentration on drug loading. There were significant effects produced when changing the drug/polymer ratio (ANOVA, $p < 0.05$). Amoxicillin of 2% w/v with a ratio of 1:2 (AMX02 and AMX05) has the lowest drug loading as compared to a drug/polymer ratio of 1:1 and 2:1.

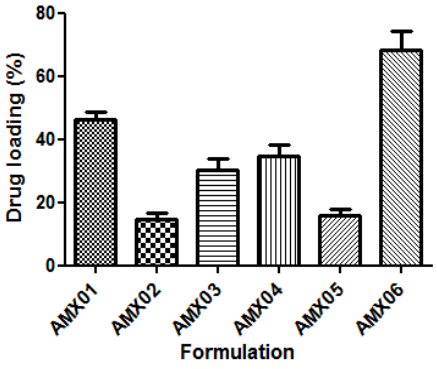

Figure 8: Comparison of Drug Loading

A higher percentage of Amoxicillin increases the chances for drug particles to be loaded into the polymeric network structure inside the beads. A higher concentration of Sodium Alginate produces a more viscous disperse solution which allowed more Amoxicillin to be loaded into the beads. When comparing AMX02 and AMX03, it was discovered that the droplets containing 4% w/v of Sodium Alginate had a greater bonding capacity for Amoxicillin in comparison to 2% w/v (*t*-test, $p >$

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0.05). The bar chart diagram (Figure 8) indicates the drug loading for the six formulations.

3.1.6 Entrapment Efficiency

The presence of $CaCl_2$ in the formulation of Chitosan coated Calcium Alginate beads provided a better entrapment efficiency of Amoxicillin in the beads (ANOVA, $p < 0.05$). 4% w/v of $CaCl_2$ produced higher entrapment efficiency as compared to 6% w/v when comparing AMX01, 80.11 ± 0.07 %, with AMX04, 58.47 ± 0.10 %. $CaCl_2$ interacts with Sodium Alginate *via* polyelectrolyte complexation to produce a gelling structure on the microspheres. A lower concentration of $CaCl_2$ will produce an instant gelling process thus entrapping a higher amount of Amoxicillin in the microspheres. The Entrapment Efficiency of Amoxicillin did not appear to be affected when the Sodium Alginate concentration was varied equally it was not affected by varying the Drug/Polymer ratio ($p < 0.05$). Figure 9 provides a comparison graph of the entrapment efficiency for all six formulations.

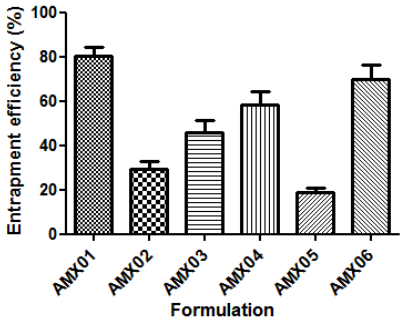

Figure 9: Comparison of Entrapment Efficiency

3.1.7 FTIR

Figure 10 displays the FTIR spectrum of Amoxicillin loaded microspheres and Table 4 is a summary of the IR bands observed in the sample. Figure 11 compares the spectrum of Amoxicillin, Sodium Alginate, Chitosan and Amoxicillin loaded microspheres.

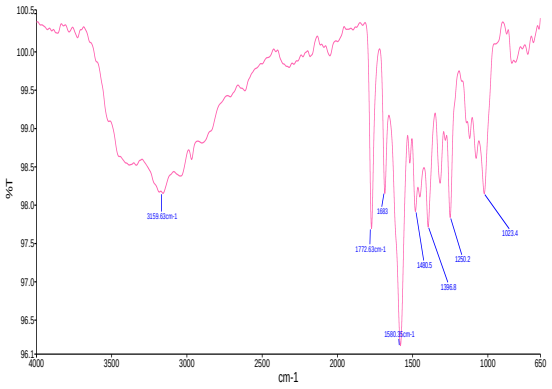

Figure 10: FTIR analysis of Amoxicillin loaded microsphere

Table 4: Summary of IR bands produced from Amoxicillin loaded microspheres spectrum produced by FTIR

Peak (cm^{-1})	Interpretation
3159	OH bonds stretching and amine group
1772	C=O of β-lactam ring
1683	C=O primary amide
1580	N-H bending vibration of amino group
1023	-O- Ether functional group

From the spectrum in Figure 10, it was noted that significant IR bands of Amoxicillin (Figure 3) are present in the microspheres: The peak at 1772 cm^{-1} in the microsphere IR spectrum corresponds with the C=O in the β-lactam ring. The second peak at 1683 cm^{-1} corresponds with the C=O amide. The peak at 3159 cm^{-1} indicates the presence of O-H and N-H stretching bonds originating in the Chitosan and Alginate polymers. The band at 1580 cm^{-1} corresponds with the N-H bonding vibrations while the band at 1023 cm^{-1} corresponds with ether linkage in the polymers. As reported by Songsurang, and researchers (2011), the band produced by the microspheres is shifted and broadened versus the pure drug indicating that there is a chemical interaction between the two. This is not observed in the spectrum of the Amoxicillin loaded microspheres in Figure 11.

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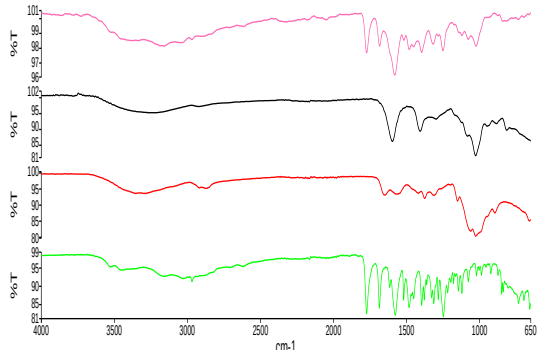

Figure 11: Infrared spectra of Amoxicillin loaded microspheres (pink), Sodium Alginate (black), Chitosan (red) and Amoxicillin (green).

3.1.8 Swelling index

The swelling capacity of the microspheres was investigated at 37°C ± 0.05 in a buffered solution of pH 1.2 to mimic stomach conditions and varying the duration of immersing at 0.5, 1, 1.5, 2 and 4 hours respectively. The swelling index values are calculated and tabulated in Table 5 and graphed on Figure 12.

Table 5: Swelling index of formulated Amoxicillin loaded microsphere (n=3)

Time (hours)	Formulation number and swelling index (%)					
	01	02	03	04	05	06
0.5	57.69	66.87	57.50	56.41	59.36	54.32
1	49.36	58.90	54.38	53.85	61.49	52.47
1.5	41.67	54.60	50.94	46.15	57.76	43.21
2	39.10	54.60	46.88	48.72	53.42	44.44
4	39.74	52.15	44.38	42.31	52.17	44.44

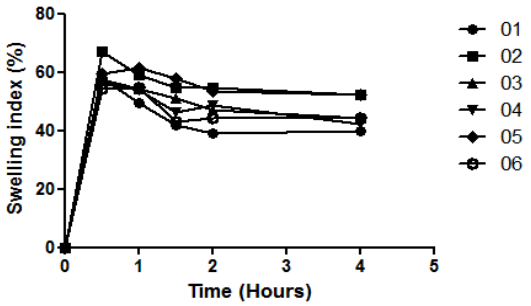

Figure 12: The swelling index graph of the different formulations of Amoxicillin loaded microspheres.

As observed in Figure 12, all formulations show rapid swelling in the first 30 minutes when exposed to the buffer of pH 1.2 and later achieve their state of equilibrium. All formulations produce a swelling index of more than 50% for the first 30 minutes. It can be concluded that the surface area does affect the swelling capacity of the microspheres. AMX05 are the smallest microspheres with the size of 35.5 ± 0.05 µm showing the highest swelling properties of 61.49%, after 1 hour exposure to the buffer medium. The samples structure remains intact after 5 hours of exposure to the buffer medium at 37 ± 0.5°C. The same graph pattern was plotted when the dissolution studies were carried out.

3.1.9 *In vitro Drug Release mechanism*

In vitro drug release studies were carried out in a buffered solution of pH 1.2. 3 ml samples were withdrawn from the dissolution bath at specific time intervals (every 15 minutes for the first hour and continuously on an hourly basis for the next 4 hours). The absorbance values of the samples were read using the UV spectrophotometer at 271 nm. A blank microsphere without Amoxicillin was measured at 271 nm. This value was used to subtract from all the results. The conversion of absorbance value to the drug release concentration was completed by inserting the absorbance value into y in the equation obtained from the calibration curve (y = 2.4553 x + 0.0059). Since the calculation was made in per 1ml, the x value obtained was multiplied by 900 ml to provide the accurate concentration of drug in the vessels.

Figure 13 graph these results and shows the drug release profile of the samples. It should be noted that only two formulations provided a release profile of more than 80% after 6 hours. The Amoxicillin released from AMX04 and AMX06 was about 89% and 80%, respectively, whereas AMX01 and AMX03 equally only released 60%. AMX02 and AMX05 have very low release profiles of 38% and 43%, respectively. All formulations produced a similar release pattern where Amoxicillin was released rapidly within 0.5 and 1 hour. It was observed that the microspheres structure remained intact and sunk in the vessels after 6 hours of dissolution testing.

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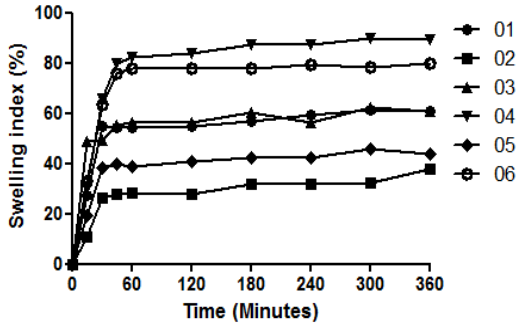

Figure 13: Drug release profile of different formulations of Amoxicillin loaded microsphere

3.2 Discussions

3.2.1 Preparation of Amoxicillin loaded microspheres

Amoxicillin loaded microspheres were successfully produced using the Ionic gelation method. The formation of the microspheres occurs when the anionic charge of the Alginate reacts with the cationic charge of the Chitosan via a polyelectrolyte interaction. The interaction between the two opposite charged polymers resulted in the precipitation of the droplets into solid spherical shaped microspheres. Chitosan is responsible for producing the polymeric matrix of the inner layer of the beads while the Calcium (Ca^{2+}) reacts with the Alginate to produce a gel layer structure on the outer layer of the beads. The Amoxicillin drug particles are entrapped in the Chitosan polymeric matrix as shown in Figure 14.

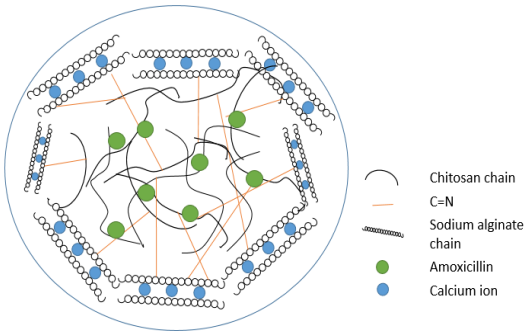

Figure 14: Illustration diagram of the Amoxicillin loaded microspheres

3.2.2 The effect of Chitosan

In this study, a medium molecular weight Chitosan with the concentration of 0.25% w/v was used to produce the Amoxicillin loaded microspheres. This is based on the result obtained from the first phase of the studies where it was found out that 0.25% w/v provided a consistent spherical structure (Razak et al., 2017). When the concentration was higher than 0.25% w/v, the structure of the microspheres was ruptured.

High concentrations of Chitosan should also increase the degree of the matrix formation in the beads and thus increase the Entrapment Efficiency of the drug. However, this also limits drug diffusion as a more compact matrix formation slows down the rate of drug release

3.2.3 The effect of Sodium Alginate

In this study, it was noted that Sodium Alginate concentration affects the Amoxicillin loading in the polymeric matrix structure. Statistical analysis (*t-test)* showed that 4% w/v of Sodium Alginate microspheres had higher drug loading when compared to 2% w/v. From our experiment, it shows that Sodium Alginate carried the drug particles into the inner layer of the beads by reacting with the Ca^{2+} ion and was not involved in the encapsulation of the beads. Therefore, increasing the concentration of Sodium Alginate should increase the chance of Amoxicillin being loaded into the microspheres.

Das and Senapati (2008) reported that increasing in Sodium Alginate concentration leads to increased entrapment efficiency due to the higher availability of Ca^{2+} to bind to the polymeric chains. However, there was no significant effect observed in the entrapment efficiency when increasing the Sodium Alginate concentration. Sodium Alginate concentration was shown that not to affect the particle size of the Amoxicillin loaded microspheres.

3.2.4 The effect of Calcium Chloride

Based on the result obtained in this study, changing $CaCl_2$ concentration did not affect the production yield of microspheres significantly. However, one parameter that was affected was the Entrapment Efficiency. It was observed that 4% w/v of $CaCl_2$ produces a better entrapment efficiency when compared to 6% w/v. It is believed that a lower concentration of $CaCl_2$ produced an instant gelling thus entrapped a higher volume of the drug particles ($p > 0.05$).

Baimark and co-authors suggest that an increase in the concentration of Ca^{2+} decreased the average

particle size and slower drug release content (Baimark & Srisuwan, 2014). However, the investigation of $CaCl_2$ in this study shows an increase in the diameter size of the microparticles when the concentration of $CaCl_2$ was increased from 4% w/v to 6% w/v. Besides that, higher concentration produced a denser gelling layer covering the Chitosan-Alginate beads thus leading to larger microsphere being produced.

3.2.5 *In vitro* Drug Release mechanism

The mechanism of drug release from the microspheres was correlated with the swelling properties of the microspheres. Pasparakis and Bouropoulos (2006) pointed out that swelling of the Calcium-Alginate microspheres was achieved in a period of time due to the equilibrium between the osmotic pressure and the forces of the crosslinking bonds that hold the structure of the polymer network. This was explain how the osmotic pressure is higher than the force of the crosslinking bond which causes the rapid release of Amoxicillin during the first 30 minutes in the study. After 30 minutes, the equilibrium occurs and the Amoxicillin is further released in a steady manner for the next five hours.

Pasparakis and Bouropoulos discussed that the amino group of Chitosan interacts with a fraction of the hydrophilic group that adheres to the surface of Calcium Alginate beads *via* a polyelectrolyte complex. When exposed to low pH media, the amino group will produce a repulsive force which leads to a swelling increment and allows water to penetrate into the beads. The hydration of the hydrophilic group causes increased weight of the Calcium Alginate beads and allow the drug particles to be release (Pasparakis & Bouropoulos, 2006).

Another reason for the rapid burst release of Amoxicillin in the first 30 minutes is due to the drug being attached on the surface of the microspheres (Liu et al., 2005). When exposed to the buffer medium, the drug particles on the surface of the microspheres were detached and released into the buffer solution.

Obeidat and Price discussed that one of the major factors influencing the drug release from microspheres is the surface area of the microspheres (Obeidat & Price, 2006). In this study, the average of microspheres surface area was lower and had a smaller particle size, thus hindering the release of the Amoxicillin from the beads. Besides that, higher water penetration is required to loosen the polymer network structure inside the beads.

The burst release profile of Amoxicillin loaded in the Calcium Alginate microspheres occurs due to the nature of the polymer and Calcium (Ca^{2+}) itself. The formation of Calcium Alginate microspheres had two major drawbacks that affect the drug release profile. (1) The formation of salts and complexation occurs when the buffer media affects the stability of the microspheres. (2) Alginate porosity causing an easy leakage of the drug particles upon contact with the buffer media (Matricardi, Meo, Coviello, & Alhaique, 2008).

These two issues can be resolved by coating the microspheres with another polymer such as Chitosan i.e. increasing the concentration of chitosan used. However, the concentration of Chitosan used in this study is not enough to protect the Ca^{2+} Alginate microspheres porosity and thus results in the burst effect for the Amoxicillin.

4. Conclusions

This study demonstrated that the Amoxicillin loaded Chitosan-Alginate microspheres can be successfully prepared using Ionic Gelation method. The results obtained are summarised as follows; (a) the microspheres produced had an average particle size between 355 ± 0.04 µm and 653 ± 0.09 µm. The particle size is greatly affected by the Amoxicillin and $CaCl_2$ concentration. (b) Raising the concentration of Amoxicillin and Sodium Alginate provides a greater drug loading on the microspheres. (c) The encapsulation of Amoxicillin in the microspheres greatly enhanced by increasing the $CaCl_2$ concentration. (d) FTIR result shows the presence of Amoxicillin peak at 1772 cm^{-1}indicating presence of the β-lactam ring and peak at 1580 cm^{-1} indicates the N-H bending of Chitosan amino group. (e) The investigation of surface morphology demonstrated high concentration of Ca^{2+} and Oxygen on the surface of microspheres suggesting that chemical reaction has been established between Chitosan and Alginate. (f) All the formulations have a swelling capacity of more than 50% upon 30 minutes of exposure to the medium and this gradually decreased. (g) Subsequently, a similar pattern was observed when a dissolution study was carried out and it was found that Amoxicillin was released in a burst release mechanism.

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