Double-Coated Nanoparticle of Ribosome Inactivating Protein (RIP) from *Mirabilis jalapa* L. prepared from Chitosan-Sodium Tripolyphosphate and Alginate-Calcium Chloride: The New Strategy for Protein Drug in Oral Delivery.

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Abstract. Oral delivery of protein drugs is challenging due to the instability of the compound and structural barrier exists in the gastrointestinal (GI) tract. Nanoparticle technology is known as a promising drug delivery strategy to ensure drug bioavailability. This study aims to formulate an oral delivery system of a potential anticancer agent named *Ribosome Inactivating Protein from Mirabilis jalapa* L.-C (RIP MJ-C) through double-coated nanoparticles prepared from Chitosan-Sodium Tripolyphosphate (TPP) and Alginate-Calcium Chloride ($CaCl_2$). Nanoparticles were prepared through the ionic gelation method, with the core nanoparticle (RMJCN-1) formulated in the pH of 3.5-5.5 using 0.3-0.5 % w/v of chitosan and 0.03 % w/v TPP. The RMJCN-1 optimum formula was selected to be subsequently coated with the second layer of alginate and $CaCl_2$, called RMJCN-2, with a concentration of 0.3% w/v and 0.1-0.3 %, respectively. The sample was characterized by the entrapment efficiency (EE), physical appearance, particle size, polydispersity index (PI), and potential zetta. The result showed the optimum RMJCN-1 formula with of EE value of 57.10 ± 0.04 % was obtained by formulating 0.5 % w/v chitosan and 0.3 % w/v STPP in pH 5.5. The optimum RMJCN-2 was obtained by the combination of alginate 0.3 % w/v and $CaCl_2$ 0.1% w/v in the outer layer. This final formula produces nanoparticles with a zeta potential of -14.4 mV, 739.8 nm in size, with good stability during 7 days at room temperature. This study has successfully developed a formulation of double-coated nanoparticles from Chitosan-TPP and Alginat-$CaCl_2$ for RIP MJ-C, leads to a safe nanocarrier system for oral delivery of RIP MJ-C that ensures its bioavailability.
Keywords: Nanoparticle, RIP MJ-C, chitosan, alginate, double coated.

1 INTRODUCTION

Protein based drug therapy is considered to be a promising strategy for cancer treatment due to its ability to selectively destroy the cancer cell without significantly damaging healthy cells [1]. Ribosome Inactivating Proteins (RIPs) is a plant enzyme having high potential as anticancer agent through ribosomes inactivation mechanism. RIPs cleavages the specific adenine *N*-glycosidic chain that causes inhibition of the prolongation factor in ribosome, leading to the termination of protein synthesis [2]. Previous research by Sudjadi et al. (2007) reported that the unbound protein fraction from *Mirabilis* jalapa L. leaves, has shown a cytotoxic effect on HeLa, myeloma, and T47D cancer cells. [3]. This unbounded protein is further known as the acidic fraction or the negative protein fraction from *Mirabilis* jalapa L., named *RIP MJ-C.*

Among all drug administration routes, oral administration is perceived as the most convenient one. However, maintaining drug bioavailability passing through the GI tract still become the main obstacles of oral drug delivery, especially for the protein-based drug. GI tract's environment may cause degradation and denaturation of proteins. In consequences, most of the peptide drugs is currently administered by parenteral routes. Developing a protein-based drug formulation that can safely permeate intestinal and cellular barriers is highly required. Here nanoparticle formulation appear as an alternative for its ability to protect compounds from premature degradation, enhancing absorbtion, regulate drug's pharmacokinetics and distribution profile, and to improve the GI tract and intracellular penetration [4] [5].

Chitosan is a cationic biopolymer that is widely used in nanoparticle preparations, and has been explored to improve the bioavailability of oral protein delivery [6]. However, the use of chitosan for oral administration is limited by its hidrophylicity and high solubility in gastric pH that might lead to drug degradation. Hence, another layer is required to protect a chitosan based nanoparticle. An alternative polymer with similar capability is sodium alginate. Sodium alginate was reported as having ability

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to deliver bioactive drugs in a sustained and controlled release manner, without risk of mucosal damage [7], [8]. Study from Bagre et al., 2013 explored a double-coated nanoparticle prepared by chitosan-TPP and alginate-$CaCl_2$ could improve the release profile and enhance the oral bioavailability of enoxaparin [9]

In this study, RIP MJ-C will be formulated into double coated nanoparticle prepared by chitosan-TPP and alginate-$CaCl_2$. This double coated design is expected to protect the protein drug from enzymatic and degradation in the GI tract. Hence, the aim of this study is to obtain a double layer nanoparticle formulation for RIP MJ-C that can maintain its bioavailability at the site of action.

2 MATERIALS AND METHODS

2.1. Materials

Mirabilis jalapa leaves crude extract was obtained through the extraction of fresh leaves in phosphate buffer pH 6.5. The unbound protein fraction of *Mirabilis jalapa* L. (RIP MJ-C) was separated from the crude extract by chromatography column using CM-Sepharose CL-6B, and confirmed by BCA Protein Assay.

Bicinchoninic Acid (BCA) Protein Assay Kit, Low Molecular Weight Chitosan (LMW-Chitosan), alginate, calcium chloride, sodium tripolypohsphate (STPP), and CM-Sepharose were purchased from Sigma-Aldrich Co.

2.2. Preparation of RMJCN-1 (the RIP MJ-C-Chitosan Nanoparticle, layer 1)

The RMJCNP-1 was prepared by ionic gelation methods. A certain amount of LMW-chitosan was stirred to dissolve in 1.5% v/v acetic acid at 50ºC. Subsequently, solution of acetate buffer with pH of 3.5; 4.5; and 5.5 respectively was added to the chitosan solution to make series of LMW-chitosan concentration of 0.3 – 0.5 % w/v for each pH. In another erlenmeyer, an amount of RIP MJ-C was mixed with chitosan and TPP with the ratio of 2:1:1 (method of Sekarningtyas, 2015) [10]. Following, the RIP MJ-C solution (0.04 % w/v) was gradually added to each chitosan solution, then homogenized by vortex for 30 seconds. Finally aqueous solution of SSTPP (0.03 % w/v) was added dropwise and mixed under vortex (50 second), leads to the formation of RIP MJ-C nanoparticle (RMJCN-1) in water. Selection for the optimum formulation was done based on the entrapment efficiency (EE) value and stability of nanoparticle formed.

2.3. Preparation of RMJCN-2 (RMJCN-1- Alginate Nanoparticle, layer 2)

The RMJCN-1 obtained form the optimum formula was separated by ultracentrifugation with RCF of 3270, for 2 hours at 4ºC. The obtained sedimen was then redispersed with water, then filtered through 0.45 µm microfilter to remove any fallout fragments and non-nanoparticle substances. In other erlenmeyer, alginate was stirred to dissolve in phosphate buffer pH 5.5 (0.2 % w/v) for 2 hours at room temperature. Series of calcium chloride solution in water were prepared with the concentration of 0,1; 0,2; and 0,3 % w/v.

The double-layered nano particle (RMJCN-2) was made from a mixture of RMJCN-1, alginate solution, and calcium chloride solution in the ratio of 1.4:9:1 respectively. The RMJCN-1 solution in water (0.5 % w/v) was added dropwise into alginate solution then mixed under stirrer for 10 minutes. Following is the addition of each concentration of calcium chloride solution dropwise, and mixing troroughly by vortex for 30 seconds.

2.4. Entrapment Efficiency Analysis (EE)

The solution of RMJCN-1 was ultracentrifugated at 15,000 rpm at 4ºC for 50 minutes. The supernatant was collected, then the existing free protein in the supernatant was measured using BCA Protein Assay Kit. A mixture of chitosan-STPP was used as the blank

Result of the BCA analysis that shows the amount of free protein, then used to calculate the %EE according to the following equation:

$$\%EE = \frac{total\ amount\ of\ protein - free\ protein}{total\ amount\ of\ protein}\ x\ 100\%$$

2.5. Visual Observation

Stability of nanoparticle was measured by observing the physical appearance of solution against a black background on the 1st, 3rd, and 7th day of storage in room temperature. Nanoparticle formation was indicated by the *opaque* solution with the absence of neither sediment nor aggregate.

2.6. Percent Transmittance (%T)

RMJCN-1 samples were analysed under UV-VIS Spectrophotometer at 610 nm. Percent transmittance determine the amount of light transmitted as it passes the solution, prescribing the presence or absense of particle agregates in the solution. Water was used as the blank sample.

2.7. Particle Size, Size Distribution and Zetta Potential Characterization

The measurement of particle size, size distribution, and zetta potential were performed with Particle Size Analyzer (HORIBA). The analysis condition was set at 24,8ºC with scattering angle of 90.

3 RESULTS AND DISCUSSIONS

3.1. Results

3.1.1. Entrapment Efficiency of RMJCPN-1

RMJCN-1 were formulated through ionic gelation technique with variations in chitosan concentration and pH environment. The calculation of %EE from formula (F1) to Formula 9 (F9) is shown in **Table 1** and **Figure 1**.

Table 1. Entrapment efficiency (EE) of RMJCN-1

Code	Medium pH	Chitosan concentration (% w/v)	EE (%)
F1	3.5	0.3	46.57 ± 0.05
F2		0.4	49.85 ± 0.04
F3		0.5	47.83 ± 0.03
F4	4.5	0.3	49.85 ± 0.02
F5		0.4	49.57 ± 0.01
F6		0.5	49.14 ± 0.04
F7	5.5	0.3	55.52 ± 0.02
F8		0.4	55.03 ± 0.01
F9		0.5	57.10 ± 0.04

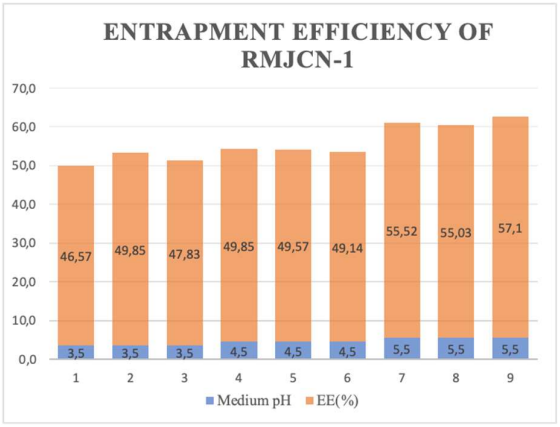

Fig. 1. Entrapment Efficiency of F1 – F9 for RMJCN-1

The EE value represents the encapsulation capacity of nanoparticle system. The high EE indicates that large amount of protein has been successfully carried in the nanocarrier system, while value low EE indicates the major loss of protein during the formulation process. Result shown in Table 1 indicate that F1 (medium pH of 3.5) has the lowest EE value (46.57 ±0.05%.), while F9 (medium pH of 5.5) was the highest (57.10 ± 0.04%).

3.1.2. Visual Observation of RMJCN-1 and RMJCN-2 Stability

The results for observations on RMJCN-1 physical stability is provided in **Table 2** below.

Table 2. Physical Stability of RMJCN-1

Code	Medium pH	Parameter	Observation Result		
			Day 1	Day 3	Day 7
F1	3.5	Agregates*	n.o.	n.o	n.o.
		%T	93.33 ± 0.31	95.06 ± 0.50	93.43 ± 0.15
F2		Agregates*	n.o.	n.o	n.o.
		%T	92.80 ± 0.80	95.36 ± 0.55	93.83 ± 0.25
F3		Agregates*	n.o.	n.o	n.o.
		%T	93.03 ± 0.60	95.33 ± 0.15	93.80 ± 0.10
F4	4.5	Agregates*	n.o.	n.o	n.o.
		%T	93.20 ± 0.44	94.50 ± 2.08	94.10 ± 0.20
F5		Agregates*	n.o.	n.o	n.o.
		%T	93.00 ± 0.36	95.63 ± 0.15	93.63 ± 0.12
F6		Agregates*	n.o.	n.o	n.o.
		%T	93.13 ± 0.31	95.60 ± 0.30	94.03 ± 0.23
F7	5.5	Agregates*	n.o.	n.o	n.o.
		%T	92.26 ± 0.32	95.16 ± 0.21	93.53 ± 0.40
F8		Agregates*	n.o.	n.o	n.o.
		%T	92.60 ± 0.40	95.10 ± 0.10	93.40 ± 0.26
F9		Agregates*	n.o.	n.o	n.o.
		%T	92.00 ± 0.20	94.50 ± 0.10	93.06 ± 0.38

*Done visual observation; n.o. = not observed; + = observed

Table 3. Physical Stability of RMJCN-2

Formula	$CaCl_2$ Concentration (% w/v)	Stability Parameter	Observation Results		
			Day 1	Day 3	Day 7
EG1	0.1	Agregates*	n.o.	n.o	n.o.
		%T	93.20 ± 0.46	93.07 ± 0.25	90.90 ± 1.31
EG2	0.2	Agregates*	n.o.	n.o	n.o.
		%T	93.73 ± 0.15	92.73 ± 1.01	90.40 ± 2,82
EG3	0.3	Agregates*	n.o.	+	+
		%T	96.03 ± 0.46	91.70 ± 1.32	93.9.0 ± 0.36

*Done by visual observation; n.o. = not observed; + = observed

The nanoparticle formation was indicated by observing the visual appearance and measuring the % Transmitance to see the turbidity of the solution. It is shown that all of the formulation depicted an *opaque* yellowish-brown liquid. The *opaque* appearance of the solution indicates the nanosize particles have been formed.

It appears that no formula formed any visible aggregates, proofing that a stable dispersion system was successfully formed. This result confirmed by the Transmittance

value from all of the samples that were above 90%. All of the NPMJ-1 formula with concentration of 0.3%-0.5% chitosan in pH of 3.5-5.5 were able to produce stable nanoparticle system in the room temperature.

From all formula for RMJCN-1, F9 (pH of 5.5 and concentration of chitosan of 0,5 %w/v) was found to be the optimum formula, giving the highest EE value and physical stability of the solution observed. This optimum formula was used for the second encapsulation step with alginate and calcium chlorida to prepare RMJCN-2.

3.1.3 Stability of RMJCN-2

The evaluation on stability of the double coated nanoparticle (RMJCN-2) was carried out as the evaluation of RMJCN-1. The results were shown in **Table 3**.

All of the RMJCN-2 show high %T value of >90%. Those indicated a low level of turbidity that would be visually observed as opaque solution. The results reveal that the difference of 0.1% w/v of calcium chloride gave no significant effect on the transmittance. Nevertheless, formula of EG3 ($CaCl_2$ of 0.3%) has shown a big fluctuation ini %T value from day 1 to day 7 of observation

3.1.4. Preparation and initial characterization of RMJCN-2

Visual observation of RMJCN-2 is shown in **Figure 3.** All formulas produce colorless and opaque solution. Based on the observation of nanoparticle physical stability, EG1 and EG2 showed stable physical appearance since during 7 days of observation. However, EG3 indicated an unstable nanoparticle system through the appearance of white aggregates which occupied ~ ½ part of solution

EG1 **EG2** **EG3**

Fig. 3. Visual observation of NPMJ-2 samples on the 7th day of observation.

3.1.5. Characterization of RPMJCN-1 and RPMJCN-2

3.1.5.1. Particle Size and Distribution

The RPMJCN-1 and RPMJCN-2 obtained form the optimum formula were characterized by its particle's diameter using *Particle Size Analyzer* (PSA), and the size distribution in the solution (*Polydispersity Index*/*PI*). These two parameters are important since they have strong correlation with homogenicity affecting bulk characteristics, product performance, processability, stability and appearance of the finished product [11], [12]. The result of the particle size analyzer and its size distribution is shown in **Table 4**.

Table 4. Particle Size and Size Distribution of RMJCN-1 and RMJCN-2

Formula	Peak No	S.P. Area Ratio (%)	Diameter (nm)	PI	Z Avr (nm)
RMJCN-1	1	62	132.1 ± 7.9	0.49	259.7
	2	38	1072 ± 69.4		
RMJCN-2	1	8	30.1 ± 1.6	0.87	521.8
	2	92	739.8 ± 45.2		

It is shown that RMJCN-1 has relative low PI (0.486) with the average particle diameter size of 259.7 nm. Further analysis of RMJCN-2 has given results of the average diameter size of 521.8 nm, and PI value of 0.870. The high PI value comes from the big difference in the diameter of the smallest (30.1 nm; 8%) and the bigest (739.8 nm; 92%) particles. The small particle presumably came from the fall out fragmen of chitosan, and the

average diameter of RMJCN-2 was detected as 739.8 nm, which still within the nanosize range and shows activity in the biological system [13].

3.1.5.2. Zetta Potential Analysis

The results of zetta potential measurement of RMJCN-1 and RMJCN-2 are shown in **Table 5** below.

Table 5. Zetta Potential Analysis Results of RMJCN-1 and RMJCN-2

Formula	Zeta Potential	Electrophoretic Mobility
RMJCN-1	+19.3 mV	0.000149 cm2/Vs
RMJCN-2	-14.4 mV	-0.000111 cm2/Vs

Zeta potensial analysis for RMJCN-1 gave a value of +19.3, which is sufficient to make repulsion force in nanoparticle system, and resulting a good stability of the nanoparticle. In the case of RMJCN-2, the zeta potenisal analysis gave value of -14.4 provides information about the existence of a negative charge on the surface of the nanoparticles.

3.2 Discussion

In the preparation of RMJCN-1, it was shown that the increase of pH environment led to gradual improvement on EE value. This might be related with the fact that electrostatic interaction is the determining factor for the association between protein - polysaccharide in nanoparticle [14], since pH will five influence on the degree of ionizaton. Formation of RMJCN-1 nanoparticles involved the protonated and deprotonated functional groups in both chitosan and RIP MJ-C. The use of medium with pH below the compound's pKa will induced the formation of protonated groups to interact with the negatively charged carboxyl group of RIP MJ-C, which also determines the strength of interaction in the nanoparticle system.

Apart from the correlation of pH environment to EE value, the effect of increasing concentration of chitosan on EE value is hardly concluded. The quantity of components involved in the reaction has an impact on the degree of interaction between each one as well as the charge or ionic group formation. The high density of chitosan fibre consequently may diminish the space for protein entrapment inside the system. Meanwhile, the low amount chitosan leads to a non-sufficient polymer quantity to encapsulate protein which contribute to the less compactness of nanoparticle system. In other words, the formation of RMJCN-1 with the best EE value could be accomplish by using a sufficient or optimum amount of chitosan theoretically. It can be concluded that the increase of pH environment gave significant effect rather than the chitosan concentration. All of the formula which is prepared in pH environment of 5.5 produced nanoparticles with good EE value (>50%), and the highest was found in the using of 0.5 % w/v chitosan.

The next step was the formulation of RMJCN-2, which was executed in pH of 5.5 as the optimum pH for RMJCN-1 preparation. RMJCN-2 is the form of double layered nanoprticles, where the second layer is constructed from alginate. In the appropriate pH medium, the carboxylate group (COO^-) of alginate will form electrostatic interaction with the protonated amine group ($-NH_3^+$) on the surface of RMJCN-1. This interaction resulted on the formation of second layer, which is strenghthened by Ca^{2+} ion from calcium chloride as the crosslinker. The predicted structure for the double layer nanoparticle RJMCN-2 is illustrated as in **Figure 2**.

From the preparation of RMJCN-2, it was found that the formulation with high concentration of calcium chloride (0.3%) resulting in nanoparticles with poor stability. This may relate to the fact found by Winarti (2011) that high concentration of crosslinker induces particles aggregation [15]. Alginate is known to be easily swell and forms gel in high temperature. These two characteristics can be mutually support the formation of aggregates in nanoparticle system.

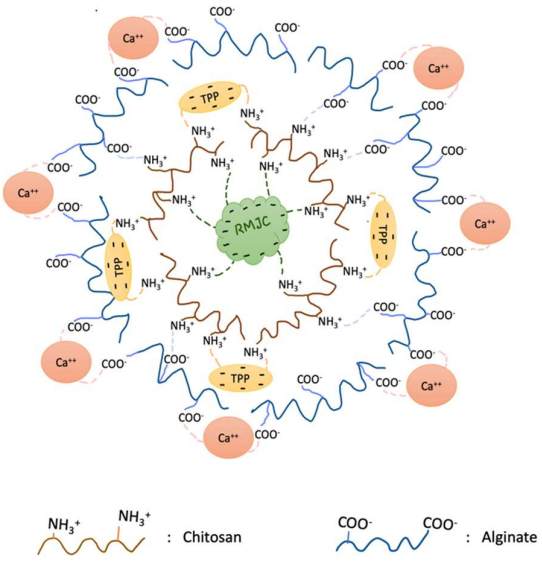

Fig. 2. Predicted complex structure for the double layer nanopartcles of RMJCN-2.

This may explain the results for EG 3, where the formation of the agreegate was likely triggered by the high concentration of calcium chloride. In lower concentration of calcium chloride (EG 1 with 0.1% and EG 2 with 0.2%), nanoparticles were more stable and showed no aggregation during 7 days of observation.

Furthermore, the tendency was more clearly seen in the transmitance tests. All of the RMJCN-2 show high %T value of >90%, indicating a low level of turbidity. Nevertheless, formula of EG3 (0.3% calcium chloride) has shown a big fluctuation ini %T value during the 7 days of observation. This phenomena may relate to the process of sedimentation in colloidal dispersion. The sedimentation began with the increasing turbidity on the surface and end with a clear appearance as the sediment went down. The transmittance value of EG1 and EG2

formula were more stable as almost no sedimentation occured. From all formula EG1 is the most stable nanoparticle, lead to conclusion that using 0.1% w/v calcium chloride for RMJCN-2 preparation a stable nanoparticle and is the optimum formula for the preparation of RMJCN-2.

In the analysis for the particle size of RMJCN-1 and RMJCN-2, it is shown that RMJCN-1 has the average particle diameter size of 259.7 nm, smaller than that of RMJCN-2 that was 521.8 nm. This bigger diameter of RMJCN-2 indicates the formation of second layer on the surface of RMJCN-1 by alginate and calcium chloride. Previous study by Ciptasari (2016) in preparing nanoparticle of RIP MJ-30 using alginate and calcium chloride gave the nanoparticle diameter range of 119-218 nm [16]. This proves that nanoparticle of RMJCN-2 represent a double coated form of nanoparticles.

Another proof of the formation of a double layer is from the results of zeta potential analysis. The results shows that the two nanoparticle systems provide different potential charges, where RMJCN-1 showed zeta potential of +19.3 mV, and for RMJCN-2 was -14.4 mV.

In a nanoparticle system with more than one component, the charges on the surface will formed with a certain value as the result of resultant ionic interaction between positive and negative charge. Chitosan is a cationic polymer with positive charge comes from ammonium group (NH_3^+). The negative charge of the protein RIP-MJC is predicted to be inside of the nanoparticle, interacts with the postive ammonium group of chitosan. Then the negative charge of STPP acts as a crosslinker to bind the positive ammonium group. Since RMJCN-1 covered with chitosan as the first layer, so the zetta potential of RMJCN-1 is postive. In the case of RMJCN-2, the zetta potential was found to be negative. This negative value is predicted caused by the carboxylate ion (COO^-) group from alginate on the outer layer. Hence, The change of zetta potential value from positive to negative in the formation of RMJCN-1 to RMJCN-2 give good indication for the success of the second layer formation by alginate and calcium chloride.

4 CONCLUSION

RIP MJ-C was successfully prepared in the form of double coated nanoparticle using chitosan-STPP dan alginate-calcium chloride. The optimum formula for first coat layer of chitosan was made from 0.5 % w/v chitosan and 0.3 % w/v TPP in medium pH of 5.5, resulting to RMJCN-1 core-particle. The optimum formula for second coat layer was made from 0.3 % v/v alginate and 0.1% w/v calcium chloride. The double-coated nanoparticles were proven to be stable in 7 days at room temperature, having zeta potential of -14.4 mV and particle size of 739.8 nm.

Further research needs to develop and to chalenge the potency of double-coated nanoparticles of RMJCN-2 as a promising drug model. Thus, the purpose of developing a stable drug delivery system for RIP MJ-C through oral administration could be accomplished.

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