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Conjugation of Anti-EpCAM Antibody on Alginate–RIP MJ-30 Nanoparticle through Carbodiimide Reaction as a Model of Targeted Protein Therapy

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ABSTRACT

Ribosome inactivating proteins from *Mirabilis jalapa* L. (RIP MJ) has shown higher cytotoxic activity when being formulated as a nanoparticle. However, the selectivity of the delivery system is also an important aspect when it comes to cytotoxic cell therapy. Epithelial cell adhesion molecule (EpCAM) is a monomeric glycoprotein which is overexpressed in epithelial cancer cells. This study aim was to develop a model of targeted protein delivery system by formulating the base fraction of RIP MJ (RIP MJ-30) into alginate nanoparticles and conjugating it with anti-EpCAM antibody. RIP MJ-30 was formulated in to nanoparticle using alginate and $CaCl_2$ as cross-linker. Optimization of conjugation reaction condition was done in the pH variation of 4.5, 5.5, and 6.5. The success of conjugation was analyzed qualitatively using native polyacrylamide gel electrophoresis (native-PAGE) method and BCA assay. The optimum formula of RIP MJ-30 nanoparticles was produced using 0.3% alginate and 0.2% $CaCl_2$. Results indicated that optimum conjugation reaction was carried out at pH level of 5.5. The optimum native-PAGE condition was by using 8% polyacrylamide gel in duration of 6h. Characterization of nanoparticle resulted in particle size of 205.0nm, zeta potential of -6.9mV, entrapment efficiency of 71.11±4.84%, and conjugation efficiency of 89.55±6.18%. It was concluded that RIP MJ-30 was successfully formulated into alginate nanoparticle and conjugated to anti-EpCAM antibody through carbodiimide reaction using 1-ethyl-(dimethylprophilamine) carbodiimide (EDAC).

Key words: Nanoparticle, bio-conjugation, EDAC, *Mirabilis jalapa,* alginate, anti-EpCAM

INTRODUCTION

Ribosome Inactivating Proteins (RIP) are proteins that usually found in plants, and have the ability to irreversibly disturb protein synthesis process. This process happened by ribosome inactivation through specific mechanism known as site-specific rRNA N-glikosidase activity (Barbieri *et al.,* 1993; Stirpe *et al.,* 2006). *Mirabilis jalapa*, locally known as 'four o'clock plant' in Indonesia, have been found to contain RIPs with anti-cancer properties (Sismindari *et al.,* 2010). Ikawati *et al.* (2006) has isolated RIP-like protein with molecular weight of 30 kDa from *M. jalapa* L. leaves, called MJ-30, which was toxic against T47D and SiHa cells (Ikawati *et al.,* 2006). However, the use of proteins as a therapeutic agent is limited by its instability, non-selectivity, and rapid elimination by enzymatic degradation (Torchilin and Lukyanov, 2003). One potential strategy to overcome this challenge is through targeted nanoparticle formulation of the protein using biopolymer, and followed by conjugation of antibody as a cancer cell targeting molecule.

Targeted nanoparticle delivery system could be made by conjugation reaction to link the

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particle surface onto a targeting molecule, with the ability to facilitate specific delivery to the targeted cells. Nanoparticles conjugated onto monoclonal antibody is an option for cancer therapy (Scott *et al.,* 2012). Previous studies have shown that nanoparticles of RIP MJ formulated with various polymers have higher cytotoxic effect compared to unformulated RIP MJ (Feranisa *et al.,* 2015). Epithelial Cell Adhesion Molecule (EpCAM) which is overexpressed in epithelial cancer cells, is a potential target molecule for targeted protein therapy for epithelial cancer. Conjugation of anti-EpCAM antibody onto RIP MJ nanoparticle have been done previously but shown only a small increase of cytotoxic effects of the protein towards T47D cells due to non-optimal conjugation process (Witjaksono *et al.,* 2016). Further study on the use of combination of chitosan and pectin as constituent, resulting in RIP MJ-anti-EpCAM nanoparticles with better entrapment efficiency but bigger diameter size (Pertiwi *et al.,* 2018). In this study we used alginate as the biopolymer having negatively charged groups, that would work well to create poly-electrolytes complex based-nanoparticle with the positively charged RIP MJ-30. The use of calcium chloride as cross linker is aimed to form RIP MJ 30-anti-EpCAM nanoparticles with desired diameter size.

The conjugation reaction between alginate nanoparticles with anti EpCAM antibody was facilitated by EDAC (1-ethyl-(dimethylprophilamine)carbodiimide). EDAC is a catalyst to facilitate the formation of amide bond between amine and carboxylate functional groups (Hermanson, 1996). Alginate has an abundance of carboxylate groups that could be activated by EDAC to react with the amine groups of the antibody. The reactivity of of both amine and carboxylate groups are known to be affected by the environment pH. Hence, the objective of this study is to obtain the optimal medium pH for the EDAC-catalyzed conjugation process. Analysis for protein-bound particle was done by Native/non-denaturing Polyacrylamide Gel Electro-phoresis (Native PAGE). Native-PAGE is a gel electrophoresis method which is generally used for separations of proteins which native form wanted to be preserved because in Native-PAGE (Wittig and Schägger, 2005). Therefore, the success of bio-conjugation of RIP-MJC nanoparticle with the targeting protein might be able to detect by native-PAGE.

MATERIAL AND METHODS

Formulation of RIP MJ-30 nanoparticle using Alginate and $CaCl_2$

Formulation of RIP MJ-30 nanoparticle was conducted based on Saraei *et al*. (2013) (Saraei *et al.,* 2013). Six hundred µL of RIP-MJ-30 solution in TRIS Buffer (Sigma; 0.015%) were added to 1.2mL of alginate (Shadong Biotech; 0.3%) solution under constant stirring, followed by the addition of $CaCl_2$ (Merck; 0.2mL) solution (0.1; 0.2; and 0.3%). RIP MJ-30 nanoparticles were formed spontaneously through poly-electrolyte complex (PEC). The nanoparticle suspension was then dialysed overnight at 4°C.

Characterization of alginate - RIP MJ-30 nanoparticle

Characteristic of nanoparticles were determined by measuring the entrapment efficiency (EE), particle size of nanoparticles using, poly-dispersity index and zeta potential. Entrapment efficiency of RIP MJ-30 alginate nanoparticles was determined by measuring the un-reacted RIP MJ-C using BCA assay kit (Sigma Aldrich). Average particle size, polydispersity index (PI) and zeta potential of nanoparticles were determined using laser dynamic light scattering using Delsa™ Nano-Submicron Particle Size and Zeta Potential Analyzer (Beckman Coulter).

Bio-conjugation of RIP-MJ 30 nano par- ticles to Anti-EpCam antibody

Nanoparticle suspension (625µL) was mixed with soulution of 0.1% EDAC (Sigma Aldrich; 190µL) using a vortex, followed by addition of anti-EpCam antibody (Abcam) solution (1750µL). MES Buffer (Merck) solution was then added to final volume of 5mL at three variants pH level: 4.5; 5.5; and 6.5. The mixture was then stirred (15min) followed by incubation (24h; 4°C) and then dialysed overnight at 4°C. The conjugated formed were then analysed using Native-PAGE kit (Sigma Aldrich) and and BCA assay. Result of BCA assay was used to measure the Conjugation Efficiency with following equation:

$$\text{Conjugation efficiency (\%)} = 100\% \text{ X } \frac{A - B}{A}$$

A = Amount of total antibody; B = Amount of the free antibody

Electrophoresis Native-PAGE system bio-conjugation detection

Conjugated nanoparticles (40µL) were mixed with loading TRIS buffer (10µL).

Electrophoresis was carried out in 1 x TBE buffer in polyacrylamide gel (8%), with the time of 6h. The visualization step was carried out with silver staining, then quantitative analysis for the band intensity was done by using the Image J-software.

RESULTS AND DISCUSSION

Alginate-RIP-MJ 30 nanoparticles

The nanoparticles were formed through Polyelectrolyte Complex (PEC) method, where the negatively charged carboxylate groups of alginates ($-COO^-$) formed numerous bonds with positively charged ammonium groups e(NH^{3+}) in RIP MJ-30. In this nanoparticle formulation, Ca^{2+} from $CaCl_2$ cross-linked free carboxylate groups ($-COO^-$) of alginates, encapsulating the RIP MJ-30 inside the polymer walls of alginate (Figure 1).

Entrapment efficiency (EE) of each formula was determined through BCA assay (Table I). The statistical analysis do not show any significant different between the data values. However, it was revelaed that concentration of 0.2% $CaCl_2$ resulted in nanoparticle with the highest percentage of EE (71.11% compared to 67.12% and 47.97%).

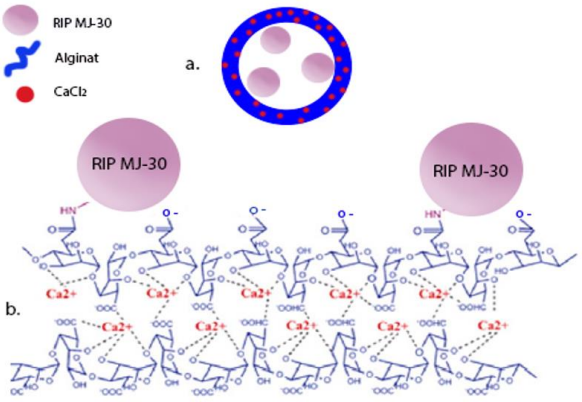

Figure 1. Predicted nanoparticle polyelectrolyte complex (PEC) formation of RIP MJ-30, alginate, and calcium chloride

The lower EE value in nanoparticle formulated with 0.1% $CaCl_2$ is assumed due to the fact that there was not enough Ca^{2+} to form the nanoparticle entrapping RIP MJ-30. On the other hand, increasing the concentration of $CaCl_2$ from 0.2% to 0.3% unexpectedly caused a significant drop of EE value. It seemed that with concentration of 0.3%, an excess of Ca^{2+} might competes with RIP MJ-30 to bind with alginate, caused the lowering the amount of RIP MJ-30-alginate complex.

Table I. Entrapment Efficiency (%) of nano-particle formulation

$CaCl_2$ Concentration (%)	Entrapment Efficiency (%)
0.1	67.12
0.2	71.11
0.3	47.97

Characterization of Alginate - RIP MJ-30 nanoparticle

Particles obtained from the optimum formula of RIP MJ-30 alginate having size between 187.4-218.60nm with an average of 153.7nm and zetta potential of -6.9mV. The polydispersity index (PI) of 0.279 showed that the nanoparticle system has a good uniformity in particle size. Particle size is an important parameter in drug delivery system because they affect the loading capacity, drug release process, and stability of the nanoparticle (Fang *et al.,* 2006). According to Gupta (2006) and Lu *et al.,* (2009), nanoparticle with the size of 280 nm or lower could be applied to deliver drugs through capillary blood vessel (Moharanj *et al*., 2006; Gupta *et al.,* 2006). Therefore, the resulted RIP MJ-30 alginate nanoparticle had an appropriate particle size to be developed as a targeted anti-cancer drug.

Bio-conjugation of RIP-MJ 30 Nanoparticle to Anti-EpCam antibody

Optimization of pH condition for the conjugation process was carried out in three pH variants: 4.5, 5.5, and 6.5 (the optimum pH medium range for EDAC reaction (Ranjan *et al.,* 2012). The optimum condition was firstly studied using free alginate to conjugate with Anti EpCAM antibody. Analysis was done using BCA reagent to detect the unconjugated antibody in the sample. The detected amount of free anti-EpCam antibody was then used to calculate the percentage value of conjugation efficiency using equation in section 2.3. The result of the conjugation reaction between the free alginate with anti-EpCAM (Table II).

Table II. Conjugation efficiency at various medium pH (n=3)

Medium pH	Un-conjugated Antibody (%)	Conjugation Efficiency (%)
4.5	43.5±8.74	56.49±8.78
5.5	13.8±7.71	89.55±6.18
6.5	32.5±5.97	67.45±6.02

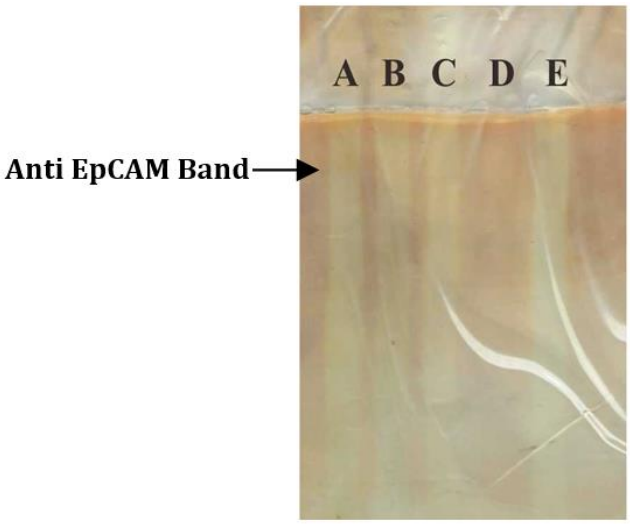

Figure 2. Gel Electrophoresis native-PAGE samples in pH of 4.5 (A), 5.5 (B), 6.5 (C). D is control for antibody anti-EpCAM, E is control for sodium alginate

Table III. Conjugation Efficiency measured Image-J software of Protein Band intensity form electrophoresis result

pH	Efficiency (%)
4.5	83.86±0.29
5.5	87.13±0.39
6.5	84.65±0.27

The results indicated that out of the three samples, pH level of 5.5 showed the best value of conjugation efficiency, which was 89.55±6.18. Statistical analysis confirmed the significant differences between the data groups (t value <4.03, $p<0.05$, n=3).

To confirm the result of the BCA assay, further analysis was done by using native-PAGE electrophoresis to separate and measure the un-reacted antibody (Figure 2). The effectiveness of the conjugation reaction in every pH medium was then analyzed by comparing the protein band intensity using *Image-J software.* Result of intensity calculation (Table III), reveals that the pH level of 5.5 is the best condition for the bioconjugation reaction. Statistical analysis confirmed that there is significant differences between the data groups of efficiency (t value <4.03, $p<0.05$, n=3).

The role of EDAC in the reaction is to form an active intermediate O-acylurea to react with amine groups in anti-EpCAM antibody, which was initiated by protonation of EDAC catalyst. Hence, an optimum pH was needed to facilitate the active intermediate to undergo the reaction. The result shown that conjugation at medium pH of 4.5 and 6.5 resulting in low efficiency. This was suspected due to less protonation of the intermediate at pH of 6.5, while at pH 4.5 the protonation of amine groups occurred and caused lost of the amines reactivity.

Based on the result, the conjugation reaction of RIP MJ-30 nanoparticle with anti Ep-CAM was conducted at medium pH of 5.5. This conjugation resulting in the form of new nanoparticles with diameter size of 205.0nm. The diameter of new nanoparticles' increased by 46.7nm, from 153.7nm to 205.0nm. Since IgG antibodies are generally 20-40nm in length so the size of the conjugated nanoparticle should be 173.7-193.7nm (Chen *et al*., 2004), lead to the supposition that nanoparticle of RIP MJ 30-anti EpCAM has been formed.

CONCLUSION

The optimum RIP MJ-30 nanoparticles was formulated using 0.3% alginate and 0.2% $CaCl_2$. The RIP MJ-30 nanoparticle was then successfully conjugated to anti-EpCAM antibody on pH level 5.5

with conjugation efficiency of 89.55%, resulting in antibody anti-EpCam-conjugated nanoparticles with diameter of 205.0nm.

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