Characterization and Prospect of Irradiated Chitosan as Nano Complex Material to Deliver MicroRNA in Cancer Therapy

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Keywords: Irradiated Chitosan; Nano complex; MicroRNA

Abstract: Chitosan is odorless white powder derived from the partial deacetylation of chitin which is a polysaccharide consisting of glucosamine and N-acetylglucosamine. Chitosan is commercially available in several types and has molecular weights that vary between 10,000 and 1,000,000. Chitosan has positively charged basic chain that can easily form nano complex with nucleic acid in this case including negatively charged microRNA. MicroRNA (miRNA) has a large role in the regulation of cancer signaling tissue so that a therapeutic approach is needed to restore the balance of dysregulated miRNA. The nature of microRNA which is very susceptible to enzyme degradation requires a special system so that it is competent to deliver microRNA into the cytoplasm. One of the factors that influence the efficiency of transfection of chitosan nano complex with a nucleic acid to body cells is molecular weight. In this research, the chitosan molecular weight reduction method was carried out to increase nano complex delivery using gamma-ray irradiation. Furthermore, characterization was carried out to determine the irradiated chitosan molecular weight using intrinsic viscosity then proceed with FTIR analysis to determine changes in chemical structure and applied further by using it in nano complex formulations with microRNA 155-p, a microRNA that experienced downregulation in ovarian cancer thus requiring mimic therapy. Results showed a decrease in chitosan molecular weight after being irradiated from 110,188 dalton to 15,209 dalton while FTIR spectra showed a break of the 1-4 glycoside bonds which was equivalent to the severance of the main chain of polysaccharides. Electrophoresis results showed that irradiated chitosan was able to form nano complex with 155-5p microRNA but transfection was not able to deliver 155-5p microRNA into the SKOV3 ovarian cancer cells.

1 INTRODUCTION

Chitosan in the form of odorless white powder derived from the partial deacetylation of chitin which is a polysaccharide consisting of glucosamine and N-acetylglucosamine. Chitosan is commercially available in several types and has molecular weights that vary between 10,000 and 1,000,000. The general function of chitosan is as a coating agent, disintegrant, film-forming agent, mucoadhesive, tablet binder, and viscosity-enhancing agent. Chitosan has been processed into several dosage forms including gels, films, beads, microspheres, tablets, and coatings for liposomes (Rowe, Sheskey, Owen, & American Pharmacists Association, 2009).

Chitosan is a polymer that has a positive charge that strong enough to form nano-sized complexes with nucleic acids that have opposite charges,

Sumadi, F., Perkasa, D., Wardana, T., Martien, R. and Harjana, S.
Characterization and Prospect of Irradiated Chitosan as Nano Complex Material to Deliver MicroRNA in Cancer Therapy.
DOI: 10.5220/0009127001910196
In *Proceedings of the 2nd Health Science International Conference (HSIC 2019)*, pages 191-196
ISBN: 978-989-758-462-6

including miRNA. MiRNA can experience upregulation and downregulation in various cancer cases (Kinose et al., 2014). MiRNA-based therapies such as mimic miRNA (artificial miRNA) are used to restore the function of miRNA that is lost due to its low expression (Tyagi et al., 2016). Chitosan-miRNA nanoparticles can increase cellular uptake by binding to negatively charged cell membranes and protecting miRNA from endogenous nuclear digestion (Chen et al., 2014).

There are several factors that affect the transfection efficiency of chitosan nanoparticles with nucleic acids such as molecular weight, degree of deacetylation, n/p ratio, nucleic acid concentration, nucleic acid dose, pH of transfection media, serum content, stability of nucleic acid and chitosan complexes, toxicity of chitosan vector, chitosan modification to facilitate transfection, and the type of cell that is transfected. The smaller molecular weight of chitosan will produce smaller chitosan-nucleic acid complexation, but chitosan with a larger molecular weight can bind plasmids more efficiently (Raftery et al., 2013)

The gamma irradiation factor (γ) used in this study to reduce the molecular weight of chitosan also includes methods for the process of sterilizing pharmaceutical products (Desai & Park, 2006). However, irradiation will affect the performance of the drug delivery system so characterization is needed to determine its properties. Determination of molecular weight is conducted to prove the irradiation process can break chemical bonds between chitosan polymers so that the molecular weight becomes smaller.

2 METHODS

Irradiated chitosan characterization

Medical grade Chitosan (PT Biotech Surindo) was dissolved became 1% chitosan solution using 100% acetic acid as much as 100 ml. Chitosan 1% solution is then irradiated with gamma-rays at a dose of 5 kGy. Then the radiation results are diluted into 0.01%, 0.05%, 0.1%, 0.15%. and 0.2% chitosan solution. Irradiated chitosan characterization is done by calculating intrinsic viscosity and FTIR test. Intrinsic viscosity is then included in the sakurada mark-houwink equation $\eta = KM\alpha$ to obtain molecular weights with K = 9.66x10-5 (dm3 / g) and $\alpha = 0.742$. FTIR testing was carried out with IR-Prestigo - ZI Shimadzu serial A210048-02492 and measured using the % transmittance measurement model, resolution = 2, apolization = hap genzel, total af.scan = 20, measurement distance = 400-4000 (cm-1) (Sionkowska et al., 2013).

Chitosan nanoparticle

0,05% chitosan solution was prepared using acetate buffer pH 5. Nanoparticles were prepared by mixing 500 ul mimic miRNA 155-5p 2 µM with 500 µl 0.05% irradiated chitosan (Martien, 2009).

Irradiated chitosan nanoparticle and transfection

40,000 SKOV3 ovarian cancer cell cultures (got from the KALBE Stem Cell and Cancer Institute) were planted on a 24-well plate that had been coated with a borosilicate glass cover. Cells were transfected with nanoparticles with miRNA that had been labeled with FAM (green fluorescence) at 4 and 48 hours. Cell nuclei were stained with DAPI blue fluorescence (4 ', 6-diamidino-2-phenylindole) (Chen et al., 2014; Ji et al., 2009)

3 RESULTS AND DISCUSSION

Irradiated chitosan characterization

High molecular weight chitosan is widely used in several biological applications. However, in many cases, the application of this polysaccharide is hampered due to high molecular weight which causes low solubility in water-based media (Czechowska-Biskup et al., 2005; Minagawa et al., 2007). Some specific applications use chitosan degradation products that are considered more useful. Some degradation methods that can be used are enzymatic, chemical or radiation. Degradation using radiation was chosen because it is simple and very environmentally friendly because it does not require initiator and by product .

Irradiated chitosan is assumed to undergo cutting off the main polysaccharide group so that it produces a smaller molecular weight compared to non-irradiated chitosan. Determination of molecular weight is conducted by finding the average molecular weight of viscosity. The results of the relative viscosity obtained using the Ostwald viscosity method. The result then processed to determine the reduction viscosity and inherent viscosity (table 1).

Table 1: Reduction viscosity and inherent viscosity of non-irradiated chitosan and 5kGy irradiated chitosan.

	Concentration (g/dL)	Flow time of sample+solvent (t) *sec*	Flow time of solvent (to)	η_r = t/to	$\eta_{sp} = \eta_r - 1$	Reduction viscosity, $\eta_{red} = \eta_{sp} / c$	$[\eta_{red}]$	ln η_r	Inherent viscosity, η_{inh}=ln η_r/c
5kGy Irradiated chitosan	0.0001	30.47111	29.166	1.044748	1.044748	10447.48	10447.48	0.043775	437.7541078
	0.0005	30.19	29.166	1.035109	0.035109	70.21875	70.21875	0.034507	69.01419285
	0.001	31.052	29.166	1.064664	0.064664	64.66434	64.66434	0.06266	62.65957125
	0.0015	31.4	29.166	1.076596	0.076596	51.06402	51.06402	0.073804	49.20283042
	0.002	32.06	29.166	1.099225	0.099225	49.61256	49.61256	0.094605	47.30274995
	0.0015	30.16	29.166	1.034081	0.034081	22.72052	22.72052	0.033513	22.34193057
	0.002	30.92	29.166	1.060139	0.060139	30.06926	30.06926	0.0584	29.19978821
Non-irradited chitosan	0.0001	31.66	29.166	1.085511	0.085511	855.1053	855.1053	0.08205	820.5040716
	0.0005	35.966	29.166	1.233148	0.233148	466.2964	466.2964	0.20957	419.1408009
	0.001	44.66	29.166	1.531235	0.531235	531.235	531.235	0.426075	426.0745991
	0.0015	54.408	29.166	1.86546	0.86546	576.9732	576.9732	0.623508	415.6717031
	0.002	64.782	29.166	2.221148	1.221148	610.574	610.574	0.798024	399.0120699

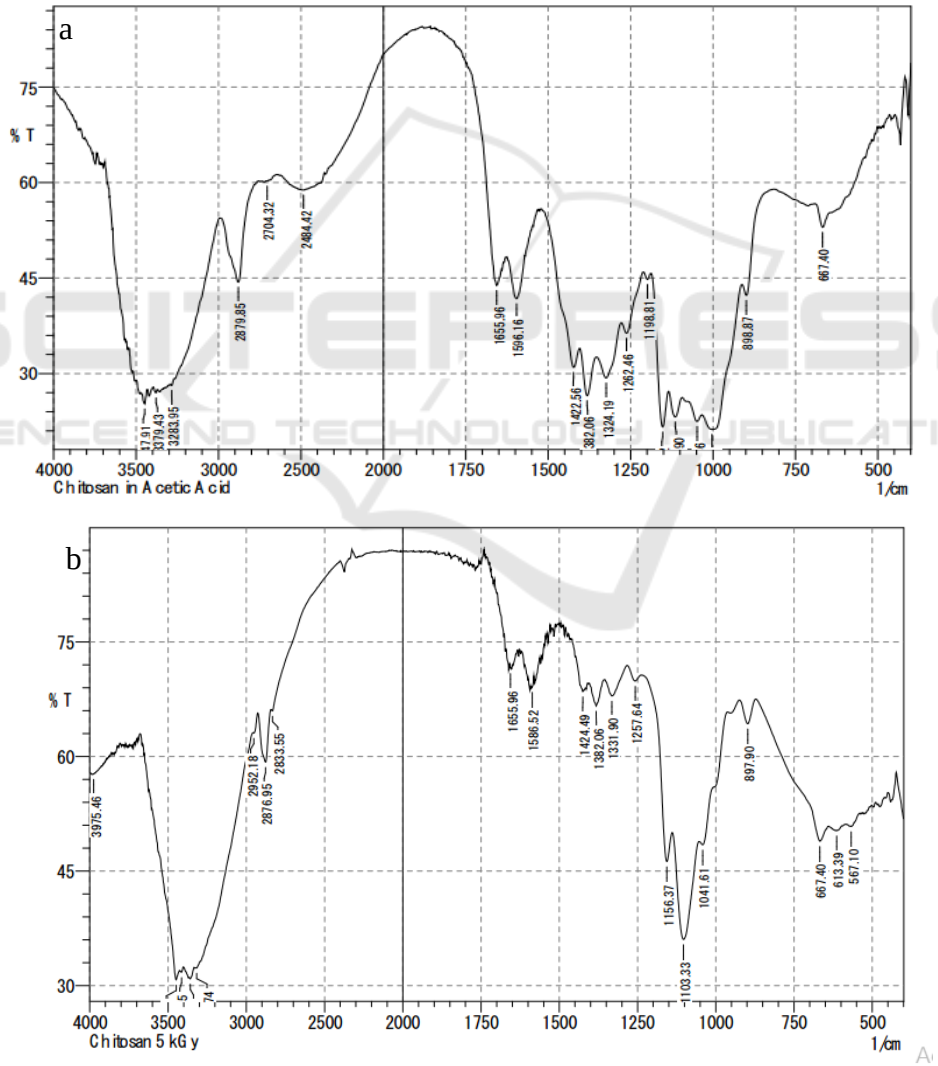

Figure 1: FTIR result of (a) non-irradiated chitosan and (b) 5kGy irradiated chitosan.

Figure 2**:** Agarose Electrophoresis Inhibition Test (a) naked miRNA-155 5p (b) Nanoparticle Chitosan irradiated-mimic miRNA 155-5p

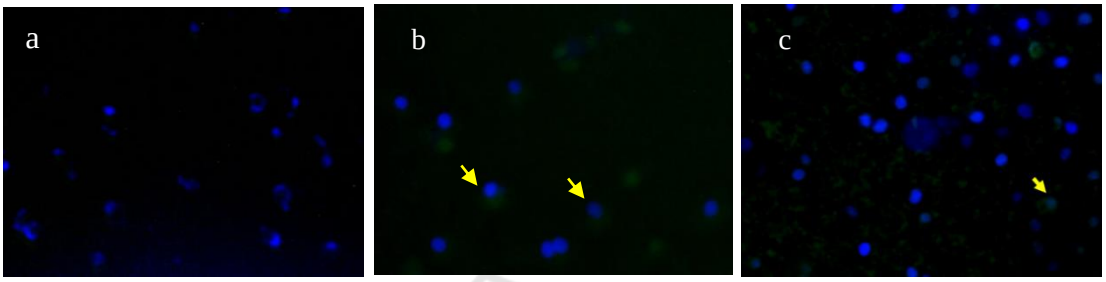

Figure 3: Transfection result of irradiated chitosan-miRNA nanoparticle to SKOV3 cell culture. (a) naked miRNA; (b) irradiated chitosan-miRNA nanoparticle after 4 hours transfection; and (c) irradiated chitosan-miRNA nanoparticle after 48 hours transfection.

The intercept results from reduction viscosity and inherent viscosity is intrinsic viscosity (η) which is then calculated in the Mark-Houwink Sakurada equation $\eta = KM^{\alpha}$ with the constant for chitosan K = 9.66x10-5 (dm^3 / g) and $^{\alpha}$ = 0.742 to obtain average molecular weight. The result shows the average molecular weight of non-irradiated chitosan is 110,188 Dalton while the average molecular weight for 5 kGy irradiated chitosan is 15,209 Dalton.

FTIR method is used to identify changes in chemical groups that occur in irradiated chitosan. From the spectra, we obtained a strong peak absorption in 1650 cm^{-1} area which shows the presence of carbonyl groups (fig 1a and 1b). There are also OH groups illustrated with peaks in the 2800 cm^{-1} region. The ester group (C-O) is also found by absorption in the 1100 and 1198 cm^{-1} regions. Meanwhile, phenol alcohol groups were seen from wide absorption in the area of 3300-3400 cm^{-1}. The ether group (C-O) can be seen from the absorption in the area of 1100 to 1300 cm^{-1}. The amino group is seen from a double peak in an area of about 3400 cm^{-1} (Silverstein et al., 2005). The significance of irradiated and non-irradiated chitosan spectra can be seen from the decrease in peak absorption in the region of 1100-1300 cm^{-1}.

Meanwhile, the uptake in the area of 1650 cm^{-1} shows both maintain their aliphatic structure. The results of the FTIR (Fourier Transform Infra-Red) spectrophotometer between irradiated chitosan and nonradiated chitosan showed similar peaks.

Identification of chitosan irradiation spectra that is identical at the characteristic peaks shows that there are no new chemical groups formed by gamma irradiation. It can be said that gamma irradiation does not induce cross-binding processes between chitosan molecules. Although high energy gamma radiation can react with chitosan groups that form free radical groups, these high-energy groups cannot easily interact because chitosan is at a solid level (Desai & Park, 2006). The absorption of ionizing radiation agents causes the localization of radical elements in the carbon atoms C1 and C4, thus breaking the glycoside bonds 1-4 which is equivalent to breaking the main chain of the polysaccharide. This can be seen clearly from the reduction in irradiation chitosan peak spectra at 1100 cm^{-1} to 1300 cm^{-1} (fig 1a and 1b) compared to irradiated chitosan which is a picture of the presence of C-O groups (Rosiak et al., 1992). This scheme is compatible with the polysaccharide degradation scheme which causes a decrease in molecular weight in chitosan irradiation.

Chitosan nanoparticle and efficacy of miRNA transfection

The MiR-155-5p expression is known to experience downregulation in advanced stages of ovarian cancer compared to early stages and followed by upregulation of HIF1α mRNA expression (Chasanah et al., 2016). Mimic-miRNA is given to ovarian cancer cells to improve the dysregulation of miR-155 5p on SKOV3.

The results of the agarose electrophoresis inhibition test showed that mimic-miR 155-5p trapped in irradiated chitosan (fig.2) to form nanoparticles so that there is no free miRNA band. This shows that nano complex can be formed between mimic-miR-155-5p with irradiated chitosan (Kaban et al., 2017; Martien, 2009; Wu et al., 2016).

The nanoparticle uptake test in SKOV3 cell culture was carried out by transfecting mimic-miRNA 155-5p nanoparticles for 2 different period, 4 hours (fig. 3b) and 48 hours (fig. 3c). The irradiated chitosan-mimic miRNA 155-5p nanoparticle transfection test was conducted by observing whether there was an accumulation of mimic-miRNA 155-5p green fluorescence around the SKOV3 cell nucleus that had been stained with blue dye DAPI. The nanoparticle formula showed that at least 155-5p mimic-miRNA entered the cell at the 4^{th} hour of transfection and decreased at the 48^{th} hour.

Particle size and charge from the surface of the nanoparticles play an important role in the uptake of nanoparticles to the cell and the efficiency of the transfection system (Wu et al., 2016). Chitosan irradiation could reduce the surface charge of the particles due to amino group termination during the radiation process. The results of FTIR showed the bias of the absorption of amino groups showed in the region 3300-3400 cm^{-1}. This shows that during the irradiation process there was an interruption of the NH_3^+ group which caused the chitosan to lose its positive charge.

4 CONCLUSIONS

5kGy gamma-rays irradiated chitosan showed a reduction in molecular weight so that the viscosity of chitosan solution was decrease than non-irradiated chitosan. Irradiation indicates a break in the main polysaccharides chain of chitosan. mimic-miRNA 155-5p nanoparticle formulation with irradiated chitosan showed good results in the electrophoresis test but not in transfection test. Irradiated chitosan nanoparticles were not able to bring mimic-miRNA into the cell. This is possible because the irradiation process cuts off the amino group which makes the chitosan charge more negative and is unable to carry mimic-miRNA 155-5p through the positively charged cell membrane.

ACKNOWLEDGMENTS

The author thanks SCI KALBE, Center for Application of Isotopes and Radiation, National Nuclear Energy Agency, Indonesia and also LPPT UGM as the opportunity to conduct the research.

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