Bifunctional Rhodamine-tagged Polymeric Nanoparticles for Analysis of Membrane Structure and Property of Living Cells

（細胞の膜構造と機能の解明を目指したローダミン標識

高分子ナノ粒子の創製）

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A Abridged Doctoral Dissertation

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Jaebum CHO

1. General introduction

Recently, biomaging combined with various nanomaterial technologies like magnetic resonance imaging (MRI), computed tomography (CT) and optical imaging have been developed. Bioimaging technology is a crucial tool to diagnose various diseases such as cancer by providing biological, biochemical and metabolic information.[1] In particular, optical imaging is very useful for *in vitro* and *ex vivo* applications in molecular and cellular biology for its advantages like non-invasiveness and sensitivity.[2] As candidate molecules for bioimaging, glycoproteins are implicated in key processes including cellular adhesion, cell-cell communication and immune response assessment.[3-6] Glycosylation is structurally diverse post-translational modifications in organisms.[7] For example, the overexpression of sialic acid, one of anionic monosaccharides in glycoproteins on the cell surface, has been implicated in pathogenesis such as colorectal cancer.[8] The investigations indicate that the expression levels of glycans on glycoproteins can be used as diagnostic biomarkers for cancers. Hence, sialic acid is an important target molecule for therapeutic systems. Similarly, virus infection involves the recognition of sialic acid. But the relationship and mechanism of virus infection and sialic acid expression is largely unknown. In the same way as cancerous disease, it is necessary to perform pathological analyses of infected cell. Observing the expression level of glycan on the membrane is one of such analysis. In addition, changes to cellular properties such as temperature should also be understood for improved diagnosis of virus infection. As a probe for detection of sialic acids, lectins are excellent candidates. Lectins that can react with specific sialic acids are often used for profiling glycoproteins. To analyze cellular membrane and property change, rhodamine is an excellent candidate. It can be easily integrated into a polymer using its derivative, and the change of fluorescence intensity with temperature was reported in recent years.[9] So it can be used as a cellular thermometer.

2-methacryloyloxyethyl phosphorylcholine (MPC) polymers have been used in various biomedical fields due to its properties such as protein adsorption inhibition.[10-11] Among the applications, the nanoparticles based on the amphiphilic MPC polymer in conjunction with other hydrophobic polymers and quantum dots has been used as a detectable biomaterial in previous reports.[12-13]

In this research, I fabricate a novel type of bifunctional probe that can play a role as a thermal sensor as well as a bioimaging probe based on rhodamine-tagged polymeric nanoparticles for analysis of cellular membrane and property change by pathological process (Chapter 2). By fluorescence intensity comparison of the polymeric nanoparticles, the overexpression of sialic acid on the cancerous cells and the change of sialic acid expression by virus infection in real time were analyzed (Chapter 3).

2. Fabrication of the rhodamine-tagged polymeric nanoparticle as bioimaging probe

I synthesized three types of rhodamine-tagged polymer with MPC, *n*-butyl methacrylate (BMA),

nitrophenyloxycarbonyl polyethleneglycol methacrylate (MEONP), and methacryloxyethyl thiocarbonyl rhodamine B (MTR) (PMBNR) via fee radical polymerization (Figure 1). Monomer compositions were varied to analyze the size of polymeric nanoparticles with respect to the BMA ratio in polymer. The active ester group of MEONP can covalently conjugate other biomolecules such as lectins.
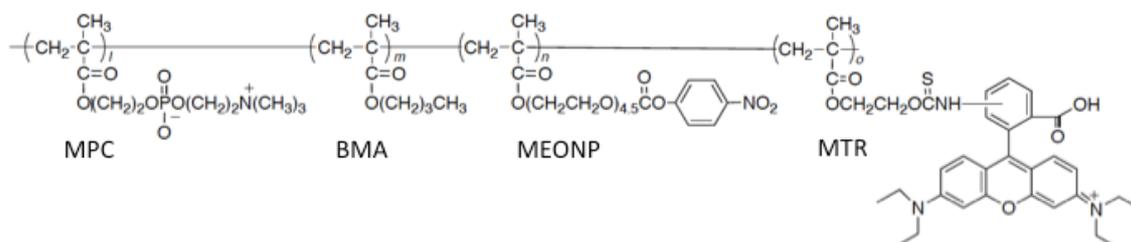
Figure 1. The structure of PMBNR.

The physical properties of synthesized polymer were measured by ^{1}H-NMR spectrum and GPC. The water-soluble PMBNR was synthesized with a similar composition to the initial feed (Table 1).

The polymeric nanoparticles were prepared by mixing PMBNR and poly(L-lactic acid) (PLA) under sonication for 5 minutes. PMBNR and PLA were dissolved in water and dichloromethane. Afterward, dichloromethane was evaporated under low pressure. The size of the fabricated nanoparticles was measure by dynamic light scattering (DLS). By changing the monomer composition ratio, the average size of the fabricated nanoparticles was controlled from 13 nm to 18 nm (Figure 2). As the BMA ratio in the polymer increased, size and the distribution also increased. It is due to the change in the hydrophobic interaction.

Table 1. The characterization of the properties of PMBNR.

	Mole fraction in feed				Mole fraction in polymer*				Yield (%)	Mw**	Mn**	Mw/Mn**
	MPC	BMA	MEONP	MTR	MPC	BMA	MEONP	MTR				
PMBNR1	0.400	0.490	0.100	0.010	0.452	0.497	0.051	- a)	88	5.8×10^4	1.3×10^4	4.46
PMBNR2	0.600	0.290	0.100	0.010	0.623	0.316	0.061	- a)	82	5.6×10^4	1.5×10^4	3.73
PMBNR3	0.700	0.190	0.100	0.010	0.756	0.199	0.045	- a)	82	6.9×10^4	1.7×10^4	4.06

a) MTR in polymer was not identified from ^{1}H-NMR because other peaks were overlapped.
*Mole fraction in polymer was determined by ^{1}H NMR spectrum.
**Molecular weight (Mw and Mn) was determined by GPC in water : methanol (v/v ratio = 3:7); PEO standard.

Also, the zeta potential of the nanoparticles on the surface was measured. For three types of the nanoparticles, the zeta potentials were near zero. It means the nanoparticles coated with MPC parts consist of zwitterion in the polymer. Among these nanoparticles, PMBNR1 nanoparticles with PLA will be used in the following experiments due to its even size distributions resulting from the strong hydrophobic interaction.
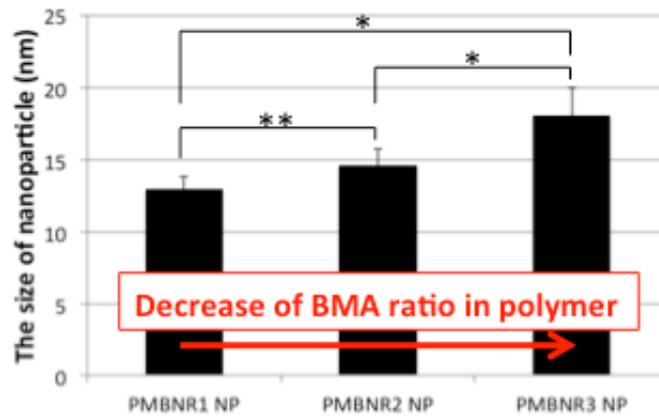
Figure 2. The size of three types of different nanoparticles by monomer compositions

To investigate the possibility as a bioimaging probe, the photostability of the nanoparticles was assessed. After 90 min of light exposure, the fluorescence intensity of the nanoparticles decreased to 30 % of non-exposed nanoparticles, while that of PMBNR1 decreased to 15 %. It suggests that the stability of nanoparticles against photobleaching increased due to the rhodamine being placed in the hydrophobic core of the nanoparticles. In the case of unshaped PMBNR1 in aqueous solution, the rhodamine exists in less hydrophobic environment. In summary, the fabricated polymeric nanoparticles possess enough photostability to be used for bioimaging probes to analyze cell membrane structure of diseased cells.

3. Application of PMBNR nanoparticles for bioimaging probes to analyze expression level of sialic acid on living cells

For fabrication of lectin-conjugated nanoparticles as bioimaging probe, streptavidin was reacted with PMBNR nanoparticles and then biotinylated lectins (*Sambucus nigra barks* lectin, which can recognize a-2,6-sialic acid) were added to the streptavidin-conjugated nanoparticles. The size of the probes was measured by DLS to be roughly 35 nm in diameter.
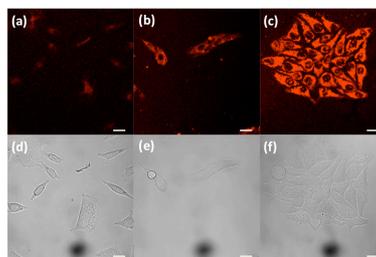
Figure 3. The fluorescence and bright field images of each cell line reacted with lectin-tagged polymeric nanoparticles ((a, d): L929, (b, e): MCF-10A, (c, f): MCF-7). (Scale bar: 20 μm)

Next, the analysis of the overexpression of sialic acid on the cancerous cell was performed. From the

reaction with the nanoparticles, it was observed that the sialic acids on the cancerous cells were overexpressed compared to normal cells (Figure 3). In the confocal images of Figure 3, the fluorescence intensity of MCF-7 was roughly 5-folds stronger than MCF10A and L929. The difference of sialic acid expression was also quantified using flow cytometer (cancer cells had roughly twice that of normal cells). It means that the lectin-tagged polymeric nanoparticles reacted more with the cancerous cells overexpressing sialic acid. In the same manner, the expression level of sialic acid was analyzed using HeLa and CCRF-CEM, other types of tumor cells. In this experiment, the expression level of sialic acid on HeLa was similar to that on MCF-7.

For confirmation of specificity of lectin for sialic acid, sialidase was used on each cell line. The result with the nanoparticle incubation indicated that there was no fluorescence on sialidase-treated cell surfaces. By removal of sialic acids on the cell surface, the nanoparticles with lectins could not be immobilized on the cells. It means lectin-conugated nanoparticles have high specificity for sialic acids on the cell membrane.
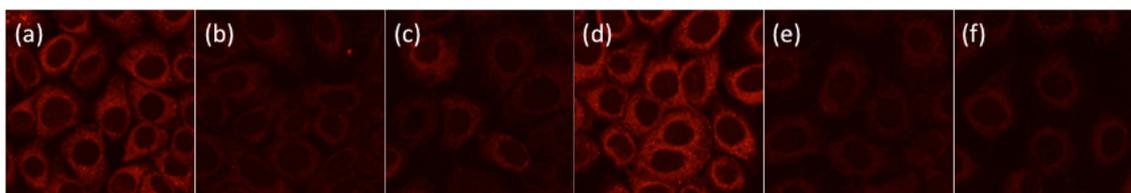
Figure 4. The fluorescence and bright field images of virus-infected cells reacted with SNA-tagged nanoparticles ((a) ~ (f): virus infected H292 cells by virus infection with 0 hour to 5 hours (from (a) to (f))

For the influenza-virus-infected cells, the analysis of sialic acid expression was performed. Before the analysis, the cells were incubated after preincubation with virus for 1 hour for the purpose of virus penetration. Subsequently, the lectin-conjugated nanoparticles were reacted to the cells in the culture dish and incubated. To analyze the change in cell membrane structure with virus infection time, the fluorescence of the nanoparticles reacted with cells was measured for 5 hours (figure 4). As a result, specific change of fluorescence intensity after virus infection could be observed. The fluorescence intensity increased after 3hours and decreased after 5 hours infection. It is presumably the change of sialic acid expression on the cell membrane by the penetration and infection mechanism of virus.

4. Concluding Remarks

In conclusion, I synthesized rhodamine-tagged polymeric nanoparticles immobilized with lectins that can recognize specific sialic acids on the cell membrane, and analyzed the expression level of sialic acid on the abnormal cells. The result showed the overexpression of sialic acid on the cancerous cells. Also, I could analyze the change of sialic acid expression level on the virus-infected cell with time. It will be

useful to investigate the virus infection mechanism. In conclusion, I believe these bifunctional nanoparticles have great potentials as a useful tool in the biomedical fields.

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