pH-Responsive Structural Change of PEGylated Amine-Bearing Nanogel Explored by Small Angle X-ray Scattering

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Abstract. A PEGylated nanogel sphere containing cross-linked amino groups in its core undergoes dramatic volumetric changes upon pH-change. We measured small angle x-ray scattering from the nanogel and analyzed the data with a core-shell sphere model to determine the core size upon pH-change from 8.0 to 5.8. The protonation of the poly-amine core due to the pH-change increased the core volume by over 300%. Combining the obtained core volume and the absolute intensity, we estimated the change of the core electron density.

1. Introduction
Poly(ethylene glycol) (PEG) modification called “PEGylation” is covalent coupling of hydrophilic PEG to pharmaceutical materials to provide prolonged blood circulation time and biocompatibility[1]. The attached PEG forms an interface which can avoid unfavourable interactions between the coated material and other biological components such as enzymes. This technology was developed from pioneering work on chemical attachment of PEG to proteins [2], and then its efficacy has been proved on polymeric micelles constructed from PEG-hydrophobic block copolymers [3], where the hydrophobic chains aggregate to form a spherical core domain in aqueous solutions. Recently Nagasaki et al [4-8] developed a core-shell structured PEGylated nanogel consisting of a cross-linked poly[2-((N,N-diethylaminoethyl)methacrylate) (PEAMA) gel and tethered PEG chains. One advantage of this nanogel over polymeric micelles is that its core can swell drastically owing to the protonation of the amino groups. This volumetric change is so drastic as to be used for endosomal escape of the nanogel and pH-stimulated drug release.

Although the biological efficiency has been shown remarkably [7, 8], its physicochemical properties are not fully understood. Dynamic light scattering (DLS) studies have shown that the sphere size at lower pH is larger than that at higher pH [8]. This pH-dependent change is reversible and repeatable. Although it seems that the gel-core plays an important role in these responses, DLS doesn’t provide detailed structural information about the core. Small angle x-ray scattering (SAXS) with synchrotron light sources is powerful tool to measure the core structure quantitatively. In the present
study, we show the SAXS results on the structural change of the core of PEGylated nanogel with a pH-change.

2. Experimental

2.1. Materials

The PEGylated nanogel sphere, consisting of a PEAMA core cross-linked by ethylene glycol dimethacrylate (EGDMA) and a PEG shell, was synthesized with a previously reported method [8]. The weight-average molecular weight ($M_w$) of the PEG chain was 7870 g/mol and the polydispersity index ($M_w/M_n$) was 1.07, both being obtained by size exclusion chromatography [8], where $M_n$ is the number-average molecular weight. The molar mixing ratio of EAMA, acetal-PEG, and EGDMA was 98.8: 1.2: 1.0. The solution pH was controlled at 5.8 and 8.0 by changing mixing ratio of 10 mM tris(hydroxymethyl)aminomethane (Tris) and 10 mM tris hydrochloride (Trizma) and was measured with a pH meter. Each solution of the sample was sonicated for 1 min using a homozinizer (SMT, UH-50) a few minutes before SAXS measurements. This treatment was found important to take out large aggregates that would cause to increase noises at low angles.

2.2. SAXS

SAXS experiments were carried out at BL03XU and BL45XU in SPring-8. An imaging plate [9] R-AXIS VII (RIGAKU, Japan) was used to measure the scattered x-ray intensity $I(q)$ as a function of the magnitude of the scattering vector $q$. Here, $q$ is described by $q = (4\pi/\lambda) \sin \theta$, where $\theta$ is the scattering angle. Two ion chambers located upstream and downstream of the sample were used to measure x-ray transmittance of the sample. To reduce parasitic scatterings, we used a specially designed vacuum chamber [10]. The sample solutions (polymer concentration $c = 2.0 \text{ mg/cm}^3$) were installed into a quartz capillary cell ($\phi = 2.0 \text{ mm}$, Hilegenberg GmbH, Germany) and then sealed with an epoxy resin. The sample to detector distances, wavelength of x-ray, and the exposure times were 4.0 and 3.5 m, 0.090 and 0.15 nm, and 30 and 300 s for BL03XU and 45XU, respectively. Wavelength and scattering vector were calibrated by the scattering of silver behenate. The obtained two-dimensional SAXS patterns of sample solutions were circularly averaged. Then experimentally determined scattering intensity profiles of the solvent were subtracted from obtained SAXS scattering profiles. Finally the differential scattering intensities were converted to the absolute intensity by use of water as a standard sample [10, 11]. SAXS intensity profiles at BL45XU ($q$-range: $0.05 < q < 0.5 \text{ nm}^{-1}$) and BL03XU ($0.02 < q < 0.1 \text{ nm}^{-1}$) were combined to cover a wider $q$-range.

SAXS experiments were performed at pH 5.8 and 8.0, since the previous DLS measurements [7, 8] showed a threshold of pH 7 in terms of hydrodynamic radius of the nanogel sphere.

To analyze the scattering data, we employed the theoretical scattering intensity of concentric core-shell spheres, which is given by:

$$I_{\text{abs}}(q) = N r_e^2 \left\{ \Delta \rho_C V_C F(q, R_c) + \Delta \rho_S V_S F(q, R_c + T_s) \right\}^2$$

(1)

where $N$ is the number of spheres per unit volume, and $r_e$ is the classical electron radius, $\Delta \rho_C = \rho_C - \rho_S$ and $\Delta \rho_S = \rho_S - \rho_0$, where $\rho_C$, $\rho_S$, $\rho_0$ are electron densities of the core, the shell, and the solvent, respectively, $R_c$ and $T_s$ are the radius of the core and the thickness of the shell, and $V_C$ and $V_S$ are the volumes of the core and the shell, respectively. For spheres, the term of $F(q,R)$ is given by $3(\sin qR - qR \cos qR)/qR^3$. As the nature of the equation (1), we do not need the absolute intensity to determine $R_c$ and $T_s$. For this case, $N$ and $\Delta \rho$ are measured in arbitrary unit. Even when the absolute intensity is given, $N$ and $\Delta \rho$ cannot be determined uniquely. In the fitting procedure we assumed that $R_c$ had a Gaussian distribution in volume ($\delta R_c$: variance of $R_c$). In addition, the effects of parasitic scatterings and dark noise of the detector were properly taken into account by adding an arbitrary constant value to equation (1).
3. Results
Figure 1 shows the measured SAXS intensity profiles and corresponding fitting curves at pH 5.8 and 8.0. It is clearly seen that there is a remarkable difference between the profiles at the different pH, showing that the size of the scattering object becomes larger with the decrease of pH. The fitting curve was obtained by the least square method using Igor Pro (WaveMetrics, USA). Due to the wide dynamic range (over three orders of magnitude) of $I(q)$, each data point was weighted by $I(q)^{-0.5}$. The fitting curves agree well with the experimental data, indicating that the present core-shell model is reasonable to describe the experimental result. The results of the fitting parameters are listed in table 1.

![Figure 1. SAXS intensity profiles at pH 5.8 and 8.0, (red and blue marks, respectively), compared with the theoretical values calculated from the equation (1) with the parameter listed in table 1.](image)

| pH     | $R_c$ (nm) | $T_s$ (nm) | $\delta R_c/R_c$ (%) |
|--------|------------|------------|----------------------|
| 5.8    | 45         | 20         | 24                   |
| 8.0    | 30         | 42         | 20                   |

As presented in table 1, $R_c$ increased by 50 % with the pH-change from 8.0 to 5.8, which means that the volume increased by over 300 %. By contrast, $T_s$ seemed to decrease by 50 % with the pH-change; this is reasonable since the core surface area increased by 2.3 times. It should be noted that the determination of $T_s$ may contain ambiguity owing to a small contrast between electron densities of water and PEG.

4. Discussion
It is important to determine the absolute values for $\rho_C$ and $\rho_S$, since they can be related to the molecular composition. We know the value of $\rho_0$ for water: $\rho_0 = 334$ e/nm$^3$. After the intensities are converted into the absolute values, we still cannot determine $\rho_C$ and $\rho_S$; we only have a multiplied value of $N(\Delta \rho_C)^2$. To resolve this problem, we used the following method: since $V_C$ is given by SAXS, the total number of electrons in the core per unit volume is related by the following equation, if we can assume that the core at pH 8.0 does not include any water.

$$N \rho_C V_C = c P_C \frac{N_{e_C} N_A}{M_C}$$

where $P_C$ is the weight ratio of the core to the whole sphere, $N_A$ is Avogadro number, $M_C$ is the molecular weight of the core, and $N_{e_C}$ is the number of electrons in the core. $P_C$ and the number of
electrons per weight, \( N_{ec}N_A/M_C \), can be calculated from the composition ratio of the sample. For two unknown parameters, we have two sets of independent equations; therefore, we can uniquely determine \( N \) and \( \rho_C \). In this way, we have determined \( N \) to be \( 1.0 \times 10^8 \) [nm\(^3\)] and the electron densities as shown in table 2. Here, the electron density of solvent was assumed to be the same with that of water, because the increase in electron density due to Cl\(^-\) in the buffer should be negligibly small.

The error of \( \rho_C \) can be evaluated from \( \delta(\rho_C)/\rho_C \), which originates from \( \delta R_C/R_c \). According to the error propagation law, \( \delta(\rho_C)/\rho_C = \delta V_C/V_C^3 = 3\delta R_C/R_c \), the errors of \( \rho_C \) are calculated as shown in table 2. We have evaluated the number of electrons transported into the core upon the pH-change on the basis of the changes of \( R_c \) and \( \rho_C \). It is \( (0.9\pm0.5) \times 10^8 \) electrons. If we assume that these electrons are transported solely by water molecules, this value corresponds to \( (0.9\pm0.5) \times 10^7 \) of water molecules. It is roughly equal to the number of the water molecules in the core at pH 5.8, \( (1.2\pm0.5) \times 10^7 \), which is estimated by assuming that partial specific volume of water in the core at pH 5.8 is the same with that of water solution. This justifies our data analysis in the present study which is based on the assumption that the core at pH 8.0 does not include any water. It should be noted that the above values are obtained to only one significant figure, which is limited mainly by the dispersity of the nanogel spheres, \( \delta R_c/R_c \). To be more precise in discussing the amount of the electron transport into/out of the core, it is required to prepare the sample which has a higher degree of monodispersity. Since \( \Delta \rho_N \) is \( ~1/10 \) of \( \Delta \rho_C \), the contribution of the shell to scattering intensity is so small that \( \rho_S \) is not evaluated precisely in the present study. The effort to enhance \( \Delta \rho_N \) is under way.

To conclude, the present SAXS work is the first to quantitatively reveal the volumetric change of the core of the nanogel accompanied with pH-change and to propose a method to evaluate the absolute electron density of the core only based on the SAXS data and the core-shell model. The validity of the obtained electron density is planned to be examined combining other methods such as static light scattering.

### Table 2. Evaluated electron densities of the core and the shell of the PEGylated nanogel sphere

|       | \( \rho_C \) (e/nm\(^3\)) | \( \rho_S \) (e/nm\(^3\)) |
|-------|----------------------|----------------------|
| pH 5.8 | 344±3                | 335                  |
| pH 8.0 | 355±7                | 335                  |

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