



Soluble Unimolecular Polymer Nanoparticles by Crosslinking of Polyethylenimine with Isophthalaldehyde

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Abstract: Polyethylenimine (PEI) is a cationic polymer widely used in non-viral gene delivery systems and other polymer technologies. PEI with a MW of 25 kD was cross-linked in aqueous solution by the aromatic di-aldehyde isophthalaldehyde. This di-aldehyde cross-links PEI by forming stable imines with the primary amine groups in branched PEI. Cross-linking was evaluated at different concentrations of PEI using NMR, dynamic light scattering (DLS or QELS) and solvent extraction. It is demonstrated that isophthalaldehyde was able to cross-link PEI intramolecularly in solution near physiological pH resulting in the formation of unimolecular cross-linked nanoparticles. Proton NMR was used to examine the cross-linking reaction *in-situ*. The aldehyde resonance of isophthalaldehyde became broadened as cross-linking occurred. Organic-aqueous liquid-liquid extraction studies supported both that the cross-linking was intramolecular and that the cross-linked structures were stable to hydrolysis in water at neutral pH. As cross-linking occurred, the percentage of isophthalaldehyde that could be extracted from the solution was reduced. The molecular radii and aggregation behavior was monitored by QELS. The particle size of the polymer molecules in solution decreased with increased cross-linking, indicating the intra-molecular cross-linking between the PEI branches. These results clearly show that cross-linking of PEI with isophthalaldehyde creates soluble unimolecular polymer nanoparticles. These materials hold promise for a variety of applications in biotechnology.

Keywords: Nanoparticles, Cross-Linking, Polyethylenimine

1. Introduction

Polyethylenimine (PEI) is a polymeric substance that has had a wide variety of applications. One common use has been as a transfection agent for importing foreign DNA into cells [1]. PEI was first investigated as a transfection vector in the 1990's [2] and has been extensively developed and exploited in this vein in the intervening years [3, 4].

In efforts to understand and implement synthetic polymers for DNA binding, a technique for cross-linking PEI as dispersed polymer molecules in aqueous solution is desirable. A preliminary report of this work was presented to the American Chemical Society [5]. The goal was to cross-link dissolved PEI molecules intramolecularly so that each cross-linked polymer molecule becomes a single polymeric nanoparticle. Since our initial study, there have been other reports of PEI cross-linking [6, 7]. The DNA binding properties and other applications of these

polymeric nanoparticles will be described in a future communication.

2. Materials and Methods

2.1. Materials

Polyethylenimine, water-free (M_w 25000), isophthalaldehyde (97%) and D₂O (99.9 atom % D) were purchased from Aldrich (St. Louis, MO, USA) and were used as received. Phosphate buffer solution was prepared with 5 mM KH₂PO₄, 5 mM K₂HPO₄, and 1mM EDTA at pH 7.0. Potassium dihydrogen phosphate, potassium monohydrogen phosphate, and EDTA disodium salt were purchased from Fisher. HPLC-grade dichloromethane was purchased from PHARMCO-AAPER. PTFE Filters (0.2

micrometer pore size) were purchased from Millipore Corporation.

2.2. Polymer Cross-Linking Reactions

2.2.1. Intra-molecular Cross-Linking in Solution

An aqueous stock solution saturated with isophthalaldehyde was prepared by adding isophthalaldehyde to deionized water until it no longer dissolved, then filtering through filter paper to remove undissolved material. PEI solutions were prepared at different concentrations as required. The PEI solutions were added dropwise to the saturated isophthalaldehyde solution. The resulting mixtures were stirred for 2 hours after addition was complete. Each solution was then precipitated by addition of 2-propanol. The precipitated solid was air dried then re-dissolved as required in DI water.

2.2.2. Inter-molecular Cross-Linking in the Solid State

The solid state samples were prepared by transferring 1 g of water free 25 kD polyethylenimine into a small beaker and adding 0.1 g of isophthalaldehyde. The mixture was placed in a water bath and stirred for 15 minutes at 100°C. The resulting product had a yellow color. To test the solubility in water, 100 mL of water was added to the cross-linked product and stirred overnight. No dissolution was observed.

2.2.3. Cross-Linking by In-situ NMR

The saturated isophthalaldehyde solutions for *in situ* ^1H NMR studies were prepared by adding an excess of isophthalaldehyde to D_2O , and removing the excess by filtration. A second solution was prepared of polyethylenimine in D_2O . The clear individual solutions were used for the standard ^1H NMR spectra of the individual species. In order to identify the optimum PEI/isophthalaldehyde ratio, the concentration of the PEI solution was adjusted by addition of D_2O . The ratio of PEI to isophthalaldehyde is given as mole:mole, with PEI determined as monomer, equivalent weight = 43D and isophthalaldehyde MW = 134D. Mole:mole ratios of 5:1, 15:1, 25:1, and 67:1 were prepared by mixing appropriate volumes of PEI in D_2O and isophthalaldehyde in D_2O , then examined by *in-situ* ^1H NMR. The NMR spectra were recorded on a GE 300 MHz spectrometer at different time points.

2.2.4. Organic Solvent Extraction

A stock solution of PEI was prepared at 0.0184 M, and the isophthalaldehyde stock solution was prepared at 0.0012 M, both in pH 7.0 phosphate buffer. The solutions were combined in volume ratios of PEI and isophthalaldehyde of 1:1, 3:1, 5:1 and 16:1. The equivalent mole ratios of PEI (as monomer, equivalent weight = 43D) and isophthalaldehyde (MW = 143D) were 15:1, 45:1, 75:1 and 240:1. The PEI and isophthalaldehyde solutions were mixed thoroughly and allowed to react for 1 hour. Extraction was performed by adding dichloromethane in an equal volume to the aqueous

solution. The extractions were shaken for 10 min then allowed to sit for 20 min to separate the layers. A solution with only isophthalaldehyde (no PEI) was used as a reference for extraction of isophthalaldehyde from pH 7.0 phosphate buffer. Electronic (UV-Vis) spectra of the dichloromethane extracts were measured on an Olis HP 8452 Diode Array Spectrophotometer.

2.2.5. Characterization of Particle Size by QELS

The molecular aggregation behavior was studied by quasi-elastic light scattering (QELS, also known as dynamic light scattering or DLS) to determine whether the cross-linking reaction at different concentrations and mole ratios formed intermolecular cross-links (aggregation) or intramolecular cross-links. The polyethylenimine concentrations were varied from 0.1 M to 1.8 M. Measurement of the radii of the PEI nanoparticles was performed at 6°C with an incident laser light beam of 632.8 nm at a scattering angle of 90° using a Wyatt Quasi Elastic Light Scattering, DAWN EOS, Wyatt Technology Corporation. Integration times were 5 seconds, with 30 scans averaged for each measurement.

Isophthalaldehyde added to PEI. Polyethylenimine solutions were prepared at 1.0 M in phosphate buffer solution at pH 7.0. The isophthalaldehyde solutions were prepared at 0.0032M in phosphate buffer solution. Both solutions were filtered through 0.2 micron filters and kept at 6°C. The isophthalaldehyde solution was then added to the PEI solution in portions. The ratio of isophthalaldehyde to PEI is given as the mole fraction of isophthalaldehyde, where X_{iso} is the moles of isophthalaldehyde divided by the sum of the moles of isophthalaldehyde and the moles of PEI determined as monomer (43D). The mixed solution was stirred for 10 min, followed by 2 min sonication. The molecular radius was then measured by the dynamic light scattering.

PEI added to isophthalaldehyde. Polyethylenimine solutions were prepared at 4.0 M in phosphate buffer solution at pH 7.0. The isophthalaldehyde stock solutions were prepared at 0.0004M in phosphate buffer solution. Both solutions were filtered through 0.2 micron filters and kept at 6°C. The PEI solution was added into the isophthalaldehyde stock solution at different volumes. The ratio of isophthalaldehyde to PEI is given as the mole fraction of isophthalaldehyde, where X_{iso} is the moles of isophthalaldehyde divided by the sum of the moles of isophthalaldehyde and the moles of PEI determined as monomer (43D). The mixed solutions were stirred for 10 min, followed by 2 min sonication. The molecular radius was then measured by dynamic light scattering.

3. Results

3.1. Imine Formation

In order to cross-link PEI to form soluble polymeric nanoparticles, we required a reaction that would be highly efficient, create stable cross-links, and function in aqueous

solution. Our choice of cross-linker was the difunctional aromatic aldehyde, isophthalaldehyde. Unlike acyl chlorides, the aldehydes are not susceptible to hydrolysis in water. Aldehydes react with primary amine groups as are found on branched PEI to form imines [8]. While imines prepared from

alkyl aldehydes with alkyl amines are unstable to hydrolysis in water, if either the amine or aldehyde is aromatic, the imine is stable at pH values near 7 [8]. A schematic of the imine formation is shown in Figure 1.

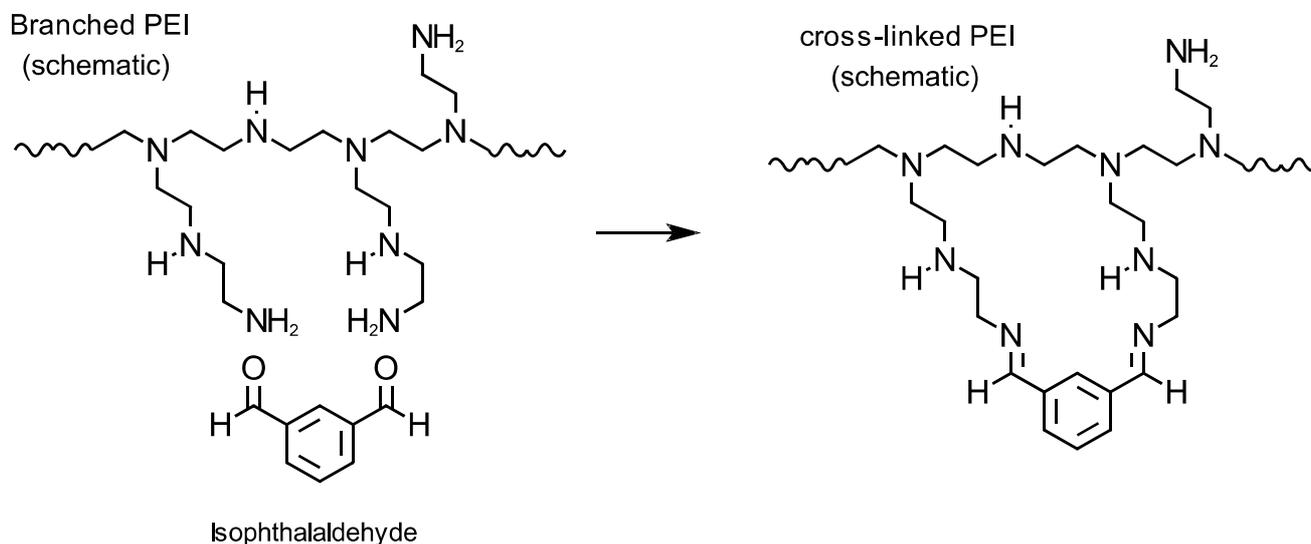


Figure 1. Schematic of cross-linking reaction. In actual branched PEI, the reactive amine ends will not necessarily be so close together.

One drawback of isophthalaldehyde is its limited solubility in water. The solubility of isophthalaldehyde is listed as “slightly soluble”, and is estimated by the EPA as 2.7 mg/mL [9]. This limits the conditions under which cross-linking can be done (PEI-isophthalaldehyde ratio), with an upper limit on the amount of isophthalaldehyde, reducing the total amount of cross-linking that can be performed. This drawback of isophthalaldehyde, while limiting, still allows for preparation of nanoparticles under a wide variety of conditions.

3.2. Solid State and Solution Cross-linking

A preliminary evaluation of cross-linking was done in the solid state and solution. In the solid state, 1 g of 25 kD PEI was treated with 0.1 g of isophthalaldehyde (this gives a PEI monomer:isophthalaldehyde ratio of 30:1, or 3.3% cross-linker) at 100°C and stirred for 15 min. A yellow color developed in the resulting solid, which was now found to be insoluble in water. This insolubility was evidence of extensive intermolecular cross-linking.

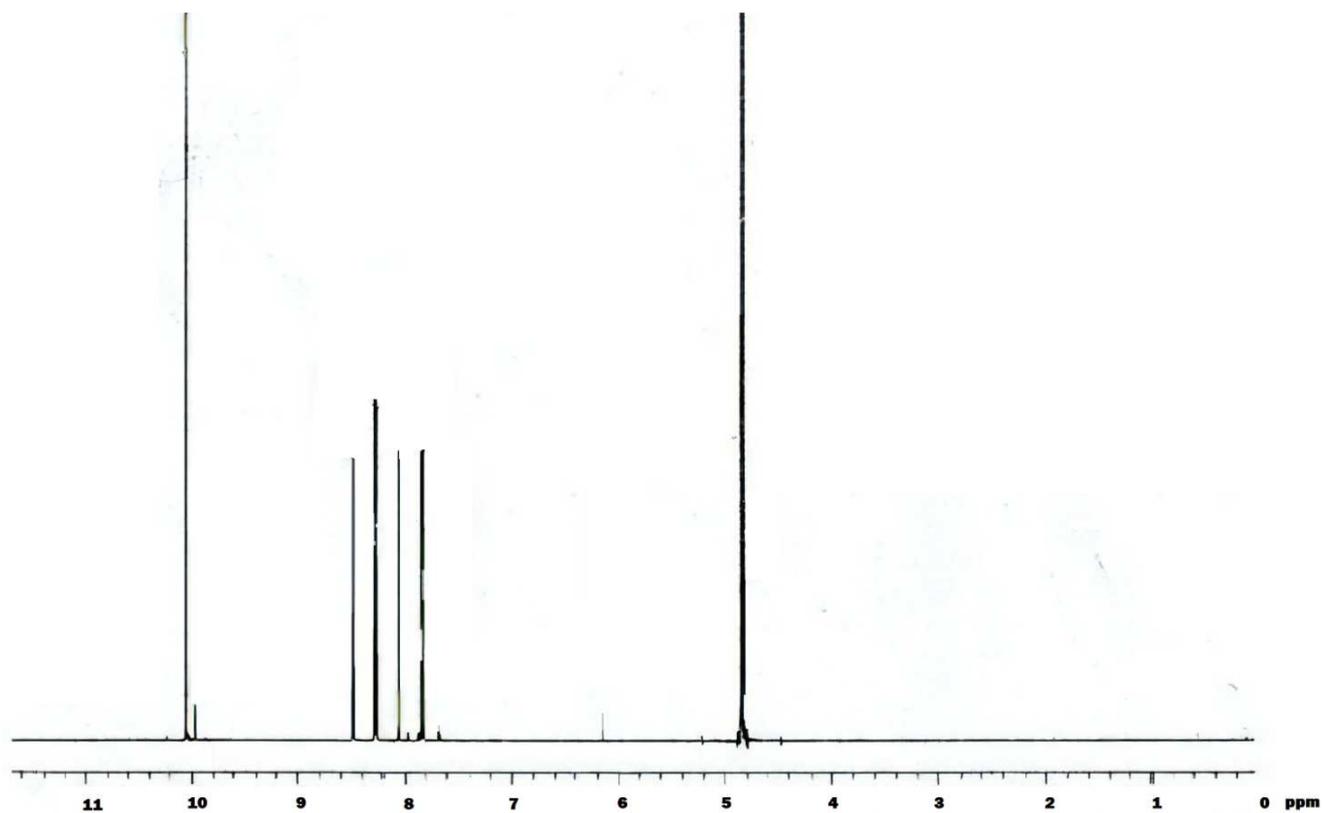
To determine if cross-linking could occur in dilute solution such that cross-links formed only within individual dissolved polymer molecules (intramolecular cross-linking), the following protocol was performed. First, a saturated solution of isophthalaldehyde (~0.0027 g/mL) was prepared in DI water. Solutions of PEI (25 kD) were prepared over a range of concentrations (0.01 g/mL up to 0.5 g/mL). The PEI solutions (5 mL) were added dropwise to the saturated isophthalaldehyde solution (5 mL), giving PEI monomer:isophthalaldehyde ratios from 11.5:1 to 575:1. The solutions were stirred for 2 hours, then precipitated with

2-propanol. The precipitated solid was filtered, air-dried, and re-dissolved in D₂O. The solid dissolved completely in water. NMR analysis was unable to observe the isophthalaldehyde, however. The ability to re-dissolve the precipitated solid suggested that cross-linking was occurring within individual polymer molecules in dilute solution, but this is not definitive evidence [10].

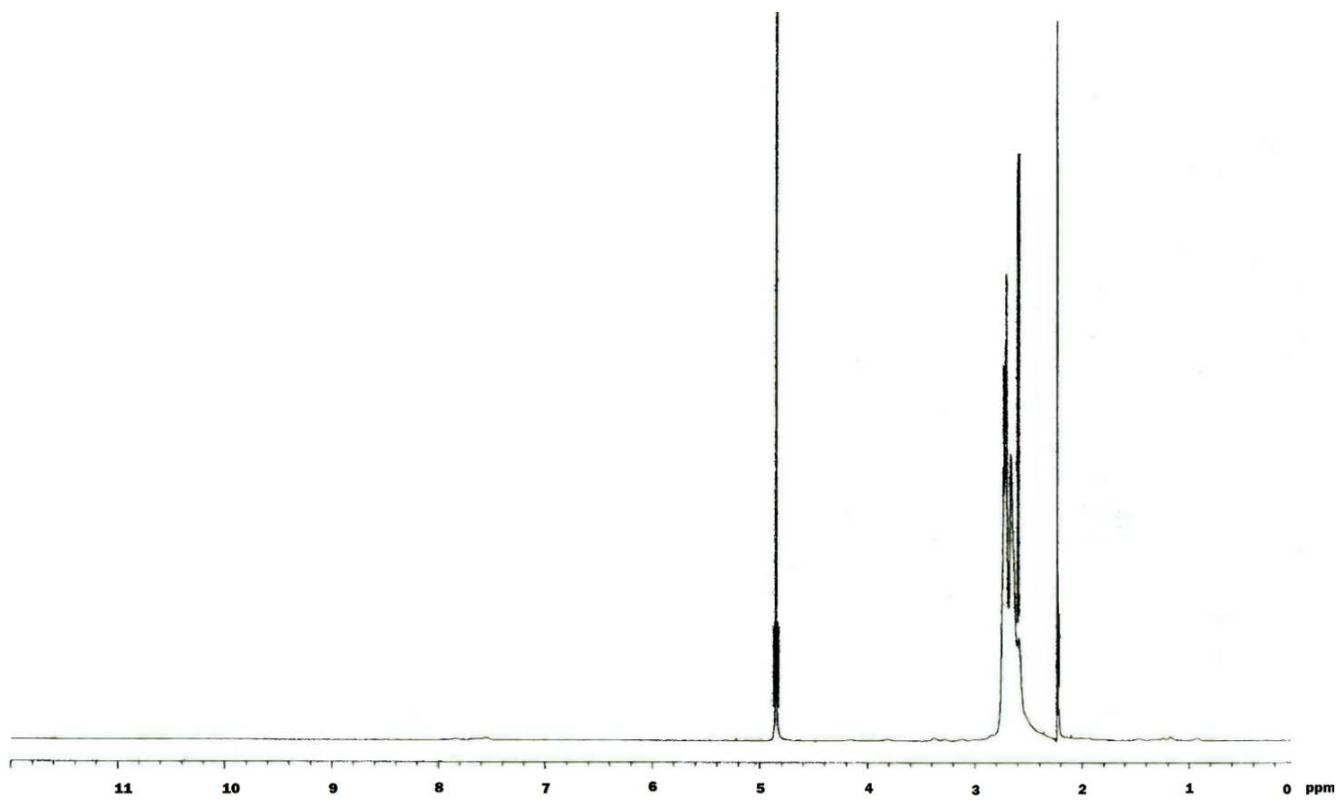
3.3. In-situ Cross-linking by NMR

To obtain clearer evidence of intra-molecular cross-linking in solution, *in-situ* NMR experiments were performed. A saturated solution of isophthalaldehyde was prepared in pH 7 phosphate buffer in D₂O, along with a solution of PEI in the same D₂O buffer.

NMR spectra of the individual components were recorded, and these are shown in Figures 2a and 2b. The solutions were then mixed to give ratios of PEI (as monomer) to isophthalaldehyde of 5:1, 15:1, 25:1, and 67:1, with upper cross-link densities of 20%, 7%, 4%, and 1.5% respectively. NMR spectra were recorded immediately after mixing and at regular intervals thereafter. Representative spectra are shown in Figures 2c (immediately after mixing) and 2d (2 hours after mixing). The isophthalaldehyde aromatic and aldehyde peaks are small due to the low concentration but are visible immediately after mixing in Figure 2c. After two hours, in Figure 2d the isophthalaldehyde peaks are broadened to the point where they are almost invisible. This is consistent with reaction of the isophthalaldehyde with the slowly tumbling polymer [11]. These experiments show that the isophthalaldehyde is linking to the PEI under these conditions.



a



b

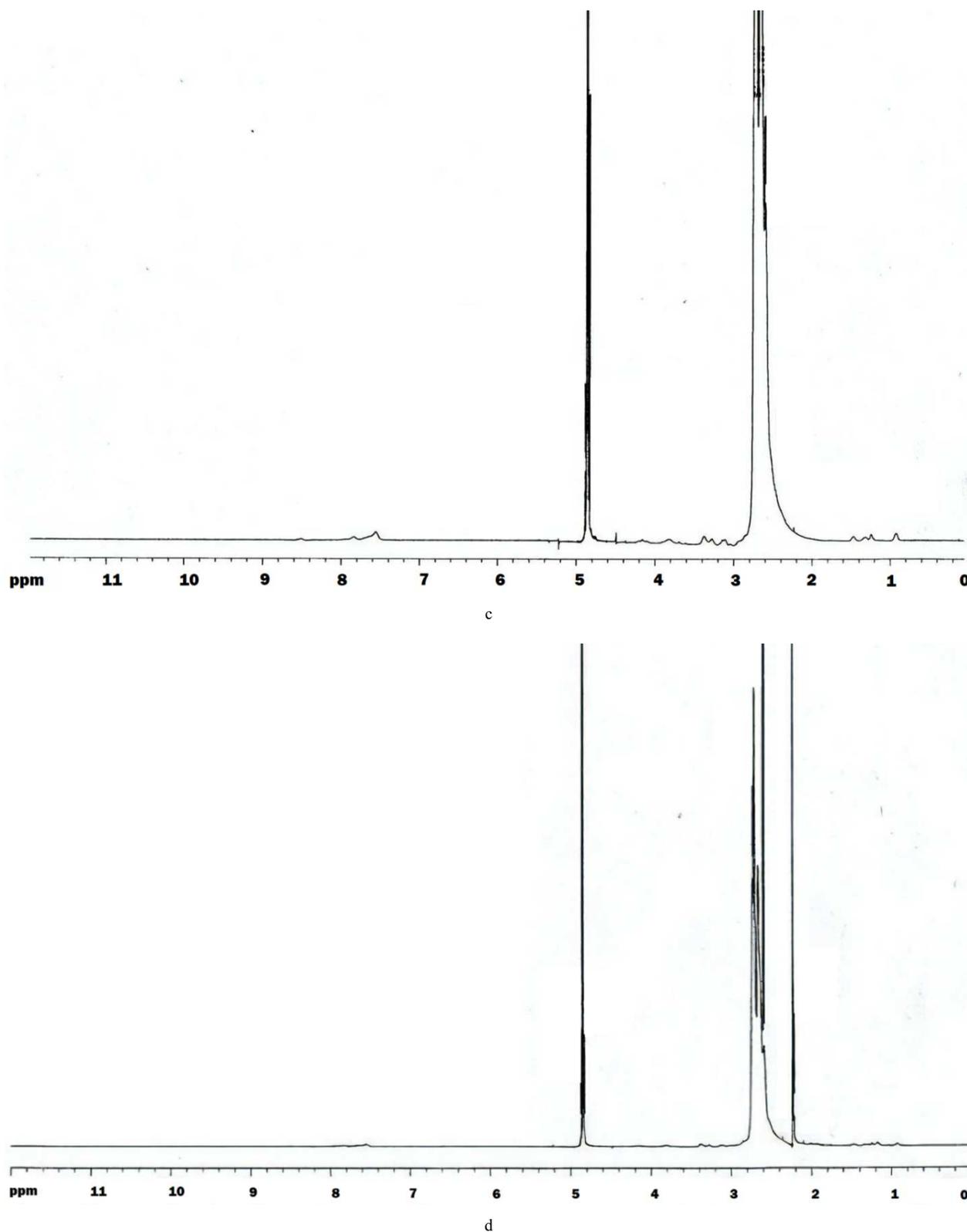


Figure 2. NMR Spectra. a. Isophthalaldehyde in D_2O . b PEI in D_2O . c. PEI-Isophthalaldehyde mixture immediately after mixing. d. PEI – Isophthalaldehyde mixture 2 hours after mixing.

3.4. Solvent Extraction

The *in-situ* NMR results showed that there was a strong association of the isophthalaldehyde with the PEI, such that

the isophthalaldehyde mobility is reduced in contact with the polymer. To demonstrate further that this association was covalent bond formation, a series of solvent extraction experiments was performed. As noted above,

isophthalaldehyde is poorly soluble in water, and much more soluble in organic solvents such as dichloromethane. Removal of isophthalaldehyde from aqueous buffer solutions by extraction with dichloromethane was monitored by UV-Vis at the isophthalaldehyde carbonyl λ_{\max} of 294 nm. When a saturated solution of isophthalaldehyde in potassium phosphate buffer was extracted by an equal volume of dichloromethane, the extraction was nearly quantitative (98% extraction). On the other hand, when a mixture of isophthalaldehyde and PEI was allowed to react at 25°C for 2 hours, then extracted with an equal volume of

dichloromethane, extraction of the isophthalaldehyde into the organic phase was greatly reduced. The amount extracted depended on the ratio of PEI to isophthalaldehyde. As the number of PEI monomer units increased relative to isophthalaldehyde, less of the isophthalaldehyde was extracted into the organic phase. Results are shown in Table 1. This is clear evidence that the isophthalaldehyde is forming covalent bonds with the PEI, preventing extraction into the organic phase. It seems logical that many of the isophthalaldehyde molecules will form two such bonds, resulting in cross-linking, but further evidence is needed.

Table 1. Extraction of PEI-Isophthalaldehyde solutions.

Ratio of PEI (as monomer) to Isophthalaldehyde	% Isophthalaldehyde extracted
0:1 (isophthalaldehyde alone)	98
5:1	36
16:1	22

3.5. Particle Sizes by QELS

The radii of the PEI molecules was measured before and after cross-linking using quasi-elastic light scattering (QELS, also known as dynamic light scattering). When cross-linking occurs in solution, there are two expected outcomes. If cross-linking is intermolecular, linking two polymer molecules in solution together, then the size of the particles will increase with cross-linking [12]. There may also be discernible population distributions of uncross-linked molecules, two cross-linked molecules, three cross-linked molecules, and so on. If cross-linking is intramolecular, it will reduce the mobility / flexibility of the polymer molecules, which in turn reduces their radii [13, 14]. Two sets of QELS data were obtained, using a high PEI concentration (1.8 M calculated by monomer equivalent weight) and a low PEI concentration (0.1 M in monomer equivalent weight). A 0.004M solution of isophthalaldehyde was added in portions, and QELS data obtained after each addition. The data are given in Tables 2 and 3 and shown in Figure 3. The hydrodynamic radius of the PEI is continually reduced with addition of the isophthalaldehyde, indicating the formation of intramolecular cross-links.

Table 2. QELS Radii of PEI with addition of Isophthalaldehyde at low PEI concentration (0.1 M).

X_{iso} ^a	Radius (nm)	Normalized Radius ^b
0	5.82	1.00
0.8×10^{-3}	5.62	0.97
2.4×10^{-3}	5.40	0.93
4.0×10^{-3}	5.13	0.88
6.4×10^{-3}	5.03	0.86
7.9×10^{-3}	4.71	0.81
11.9×10^{-3}	4.45	0.76
14.2×10^{-3}	3.89	0.66

^a X_{iso} is the mole fraction of isophthalaldehyde (iso)
= moles iso / (moles iso + moles PEI monomer)

^b Normalized radius = measured radius divided by the radius of the PEI before cross-linking (at $X_{\text{iso}} = 0$).

Table 3. QELS Radii of PEI with addition of Isophthalaldehyde at high PEI concentration (1.8 M).

X_{iso} ^a	Radius (nm)	Normalized Radius ^b
0	5.64	1.00
0.09×10^{-3}	5.53	0.98
0.2×10^{-3}	5.37	0.95
0.3×10^{-3}	4.86	0.86
0.4×10^{-3}	4.62	0.82
0.7×10^{-3}	4.53	0.80
0.9×10^{-3}	4.33	0.77
1.3×10^{-3}	4.16	0.74
1.8×10^{-3}	4.06	0.72

^a X_{iso} is the mole fraction of isophthalaldehyde (iso)
= moles iso / (moles iso + moles PEI monomer)

^b Normalized radius = measured radius divided by the radius when $X_{\text{iso}} = 0$

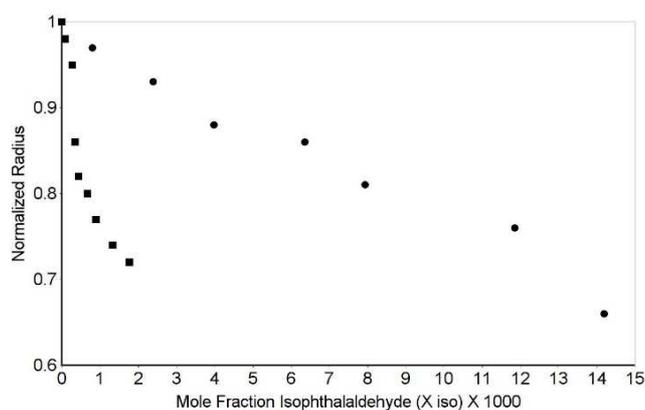


Figure 3. Normalized radius of PEI plotted against the mole fraction of isophthalaldehyde cross-linker (X_{iso}). ● Data obtained at low PEI concentrations (0.1 M PEI). ■ Data obtained at high PEI concentrations (1.8 M PEI).

4. Discussion

The goal of this research was to generate cross-linked polymer nanoparticles from polyethylenimine. The results of these experiments show that isophthalaldehyde is capable of cross-linking PEI to form cross-linked

nanoparticles. The initial tests with cross-linking in the solid state indicated that a reaction occurred, creating an insoluble cross-linked network polymer. The dilute solution experiments produced a material that could be precipitated and re-dissolved, as would be expected for an intramolecular cross-linked polymer. However, we were unable to prove that the isophthalaldehyde was contained in this material – it could have just been precipitated PEI, and the isophthalaldehyde remained in solution and did not precipitate.

The *in-situ* NMR experiments gave evidence that the isophthalaldehyde was associating with the PEI, as the spectra of isophthalaldehyde changed from sharp peaks of a freely tumbling small molecule to the broad peaks of a slowly tumbling macromolecule [11]. This was not definitive that the association was the formation of covalent bonds, as non-covalent interaction or association might also lead to slow tumbling [15].

The solvent extraction experiments gave strong, if not definitive, evidence that the association was covalent bonding. Isophthalaldehyde extraction was greatly reduced after reaction with PEI, suggesting that the interaction was through the formation of covalent bonds.

The final evidence for the formation of intramolecular covalent cross-linked particles is derived from the QELS experiments, where treatment of PEI with isophthalaldehyde led to a decrease in the particle size. Had the association been non-covalent, or non-cross linking this reduction in size would not be expected to occur [16]. If the cross-linking were intermolecular, then size would increase. The observed reduction of size by QELS is only consistent with the generation of unimolecular, cross-linked nanoparticles by intramolecular cross-linking. [13, 14, 16]

5. Conclusions

The results of this study demonstrate that branched polyethylenimine (PEI) can be cross-linked in aqueous solution by isophthalaldehyde to form unimolecular cross-linked nanoparticles at physiological pH. In this model, each nanoparticle is a single branched polyethylenimine molecule where the terminal primary amine groups have formed imine cross-links with the aromatic di-aldehyde. This conclusion is supported by the solubility of the prepared materials after precipitation, the *in-situ* NMR results and the solvent extraction results. The observation of reduced hydrodynamic radii by QELS is further proof of the intramolecular nature of the cross-linking.

These types of materials have promise for further studies in a number of directions, from the binding of nucleic acids to a variety of molecular imprinting protocols [6, 7, 17]. These research avenues will be explored in future work.

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