Enzymatic 'charging' of synthetic polymers

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Complete List of Authors:	Khan, Anzar; ETH-Zürich, Department of Materials Rao, Jingyi; ETH-Zürich,

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Jingyi Rao and Anzar Khan*

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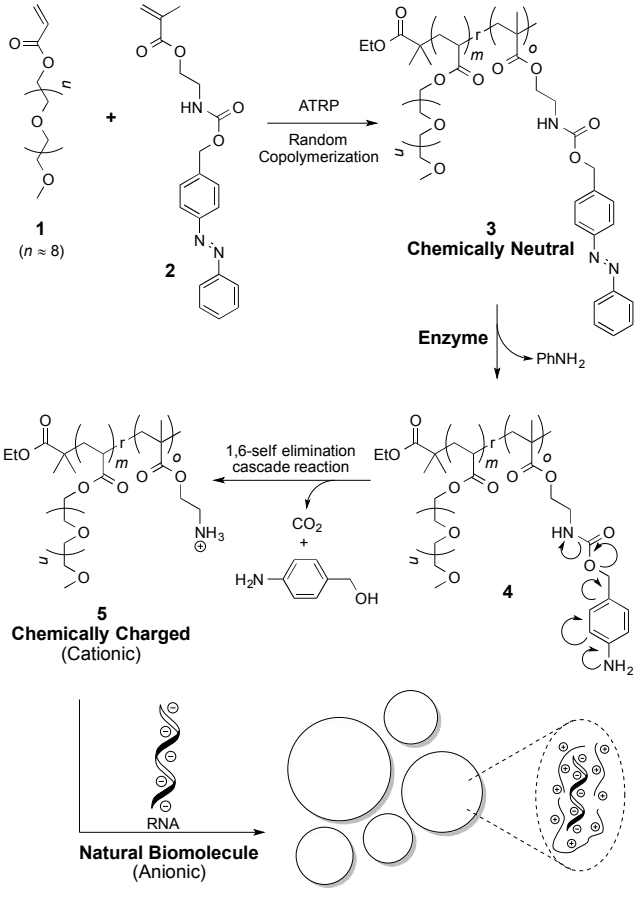
Actuated by an enzyme, a purely synthetic and chemically neutral polymer chain transforms into a chemically charged cationic structure. This biologically triggered structural change enables the polymer chain to recognize a negatively charged biomolecule (RNA) through electrostatic interactions in aqueous environment. The supramolecular recognition event ultimately leads to the assembly of oppositely charged, artificial and natural polymer chains, into the polyion complex-based nanoparticles.

Chemical transformations within a living cell are catalyzed, regulated, and facilitated (by way of providing energy) through enzymes. Inspired by this, explorations have recently begun into designing artificial structures capable of responding to an enzymatic stimulus.[1-6] A majority of the studies in this direction, however, focus on small molecules and peptide derivatives.[1-2] Synthetic polymers, with their structural versatility, synthetic ease, and tunable properties, represent attractive alternative scaffolds.[7-8] The promise and potential of these candidates in building enzyme responsive systems is already evident from various recent studies. In these studies, enzymes are used as a trigger for assembling and disassembling of well-defined polymeric nanostructures[9-13] and for construction of higher ordered assemblies.[14] With a view of practical applications, Liu and coworkers have harnessed such concepts into the development of sensory systems capable of detecting biologically relevant analytes under physiological conditions.[13] Despite these advances, it is recognized that the field of enzyme responsive polymers is still in its infancy and compared to the small molecular and peptide-based systems, few concepts have been explored in the context of synthetic polymers.[1-5] Here, we show that a carefully designed polymer chain that is water-soluble yet completely artificial in its repeat unit structure can transform from a chemically neutral to a chemically charged cationic structure upon coming in direct contact with an enzyme (Scheme 1). This enzyme triggered structural transformation of the synthetic polymer chain allows it to communicate with a negatively charged natural biopolymer (RNA) in aqueous environment. Finally, the association of the oppositely charged synthetic and natural polyelectrolytes results in an assembly process leading to the formation of nanoparticles known for their utility in the gene therapy arena.[15]

To establish the aforementioned concept, a careful design of the enzyme sensitive polymer chain was essential. At the outset, we decided to prepare a water-soluble random copolymer so that



Scheme 1. Synthesis of random copolymer **3**, its transformation into a cationic structure upon enzymatic action, and formation of nucleic acid-based polymer nanoparticles.

the enzymatic action can be carried out under aqueous conditions and the precursor polymer cannot assemble or aggregate on its own in water. For this, two (meth)acrylate-based monomers were selected (Scheme 1). Monomer **1** featured an ethylene glycol side-chain (M_n = 480, $n \approx 8$). This monomer was chosen due to the known biocompatibility and water-solubility properties of the oligoethylene glycol-based acrylate polymers.[18] Monomer **2** was designed in such a way that it carried an enzyme responsive unit – the azobenzene group – connected to a polymerizable methacrylate moiety through a carbamate linkage. This linkage, in turn, was attached to the enzyme sensitive site through a

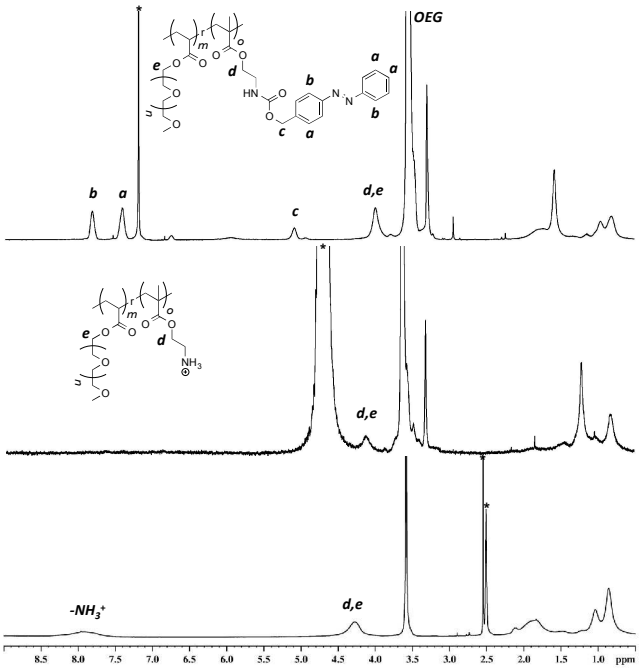


Fig 1. [1]H-NMR of polymer **3** before (top) and after the enzymatic action in deuterated water (middle) and DMSO (bottom) (OEG = oligoethylene glycol).

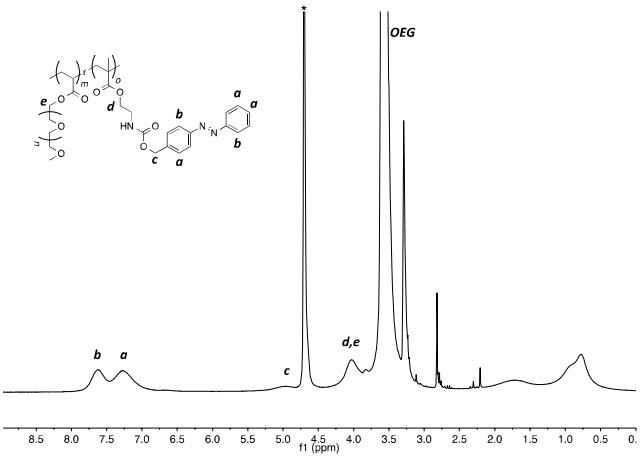


Fig 2. [1]H-NMR of polymer **3** in deuterated water.

benzylic position. From previous studies, it is known that the azobenzene group, although non-natural, can be cleaved under enzymatic conditions through a two-electron reduction process.[17,9c,10b] This can be achieved by NADPH-dependent flavoprotein enzymes azoreductase or DT-diaphorase.[19] The ultimate result of this reduction reaction is the cleavage of the azobenzene moiety into two aniline molecules. In the present case, since the azobenzene group is linked to a polymer chain, the enzymatic action would produce a polymer chain with aniline side chains. Once produced, the aniline groups can undergo a 1,6-self elimination reaction as known for such electron rich aromatic systems when a leaving group, the carbamate functionality in the present case, is present at the benzylic position to the electron donating (amine) site.[20] The motivation for such self-immolation comes from the formation of thermodynamically stable CO_2

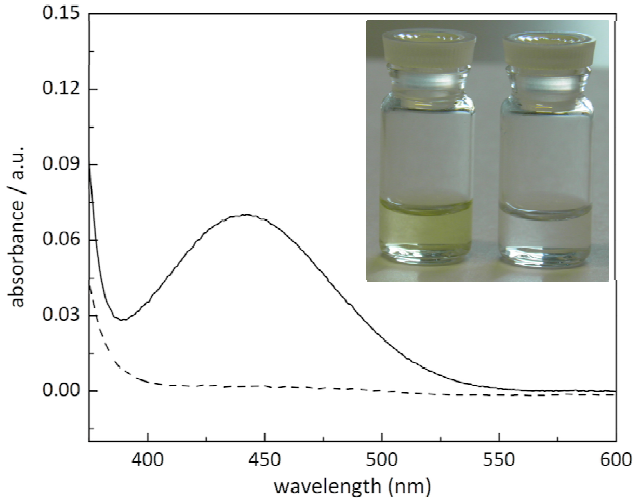


Fig 3. UV-Vis spectra of polymer **3** before (solid line) and after (dashed line) the enzymatic action in aqueous solution. The inset shows digital photograph of aqueous solution of polymer **3** before (left) and after (right) the enzymatic reaction.

molecules and an increase in the entropy of the overall system. Therefore, an enzyme triggered cascade process would generate the primary amine site at the polymer side chains. These primary amines, with pK_a in the range of 8-9, are expected to be in a protonated state in neutral aqueous solutions.[21] In this way, a purely synthetic and neutral polymer chain would be transformed into a chemically charged cationic structure upon enzymatic actuation. It is noteworthy that in the present system the structural change is brought about by direct action of an enzyme on the polymeric scaffold. In alternative systems, such changes are achieved by variation in solution pH,[22] application of light as an external stimulus,[23] or through generation of a bio-relevant analyte.[13,24] It should be mentioned that the produced aniline is a toxic entity. However, its derivatization may lead to the generation of a benign by-product.[16b]

To test this concept, monomers **1** and **2** were copolymerized in a random fashion using the atom transfer radical polymerization process.[25] The resulting copolymer **3** carried the oligo(ethylene glycol) and azobenzene moieties randomly dispersed in the polymer chain. Based on the area integration analysis ([1]H-NMR spectroscopy) using well-separated aromatic ('a' and 'b' in Figure 1) and aliphatic ('OEG' in Figure 1) signals arising from the two different polymer repeat units, random copolymer **3** ($M_{n(GPC)}$ = 142 kDa, M_w/M_n = 1.1) was established to carry a monomer ratio of 3:1 in favor of oligoethylene glycol monomer **1**. Due to this, the polymer displayed good water solubility. Furthermore, due to the random nature of the repeating units, the polymer cannot assemble into any secondary structure in water. A complete solubility of the copolymer **3** in aqueous environment was also confirmed by examining its structure through use of NMR spectroscopy in deuterated water (Figure 2). Due to the bottlebrush nature of copolymer **3** and resulting backbone stiffening, the GPC estimated molecular weight using polystyrene as a standard, is likely to be an overestimation.

To study the enzyme triggered structural change, an aqueous solution (pH = 7) of random copolymer **3** was exposed to enzyme DT-diaphorase (Human) (30 μM) along with coenzyme NADPH

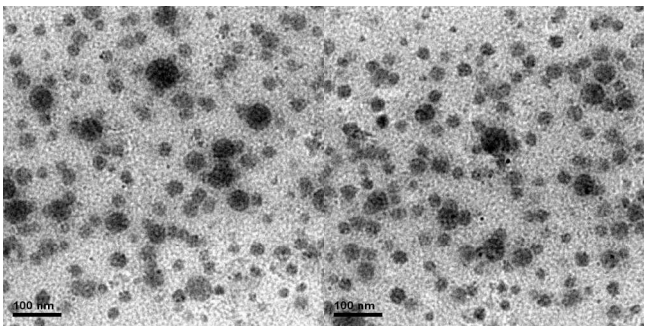


Fig 4. TEM micrographs of the nanoparticles at two different places of the TEM grid (Scale bar = 100 nm).

(0.5 mg). The mixture was incubated at 37 °C and the aqueous solution was constantly monitored with the help of UV-Vis spectroscopy. This examination revealed that the absorption from the azobenzene chromophore, located at 450 nm, disappeared after a period of 8 h of reaction time (Figure 3). This could also be observed with the naked eye as the yellow colored solution turned colorless upon enzymatic treatment of the random copolymer **3** (Figure 3). ^{1}H-NMR spectroscopy could also be used as an analytical tool. In the case of polymer **3**, aromatic proton resonances were apparent in the area of 7.3-7.9 ppm (Figure 1). However, after the enzymatic action and the cleavage of the azobenzene group, no aromatic resonances could be observed in the ^{1}H-NMR of the purified polymer **4**. To further check this, two control experiments were carried out. In one experiment, NADPH was not added to the system. In another, DT-Diaphorase was excluded. Both systems failed to convert neutral polymer **3** into cationic polymer **4** as concluded from ^{1}H-NMR and UV-Vis spectroscopy analyses. Next, to examine if the newly generated amine groups were protonated or free, the aqueous polymer solution was freeze dried and re-dissolved in deuterated dimethylsulfoxide (DMSO-d_6). In this case, a broad signal could be seen at 8 ppm. This signal could be assigned to the ammonium groups. In D_2O, this signal disappears due to a fast exchange of the ammonium protons with deuterium.[26] Structurally related polymers are known to be in a stable protonated form under neutral conditions.[13,21] In one case, even under relatively basic conditions (pH = 9), 33% of primary amine side-chains were found to be in a protonated state.[21c]

To investigate the propensity of the enzymatically generated cationic random copolymer chains towards an electrostatically driven assembly process, natural anionic polyelectrolyte, RNA derived from torula utilis (5000-8000 g/mol) (0.05 mg in 10 μL of DI water), was introduced in the aqueous system and the resulting structure was examined with the help of transmission electron microscopy (TEM) (Figure 4). This examination revealed that nanoparticles in the size range of 10-80 nm were formed in the system. This was expected due to known works in the area of synthesis of polyion complex nanoparticles.[15] To further explore this aspect, dynamic light scattering (DLS) measurements were performed. These measurements indicated that throughout the enzymatic reaction small polymer coils were present in the solution as can be gauged by the particle sizes below 5 nm (Figure 5). However, upon introduction of the nucleic acid, particles of larger size (10-150 nm) were formed. The larger sizes under DLS analysis most likely reflect upon the hydrated state of the material in aqueous solution.

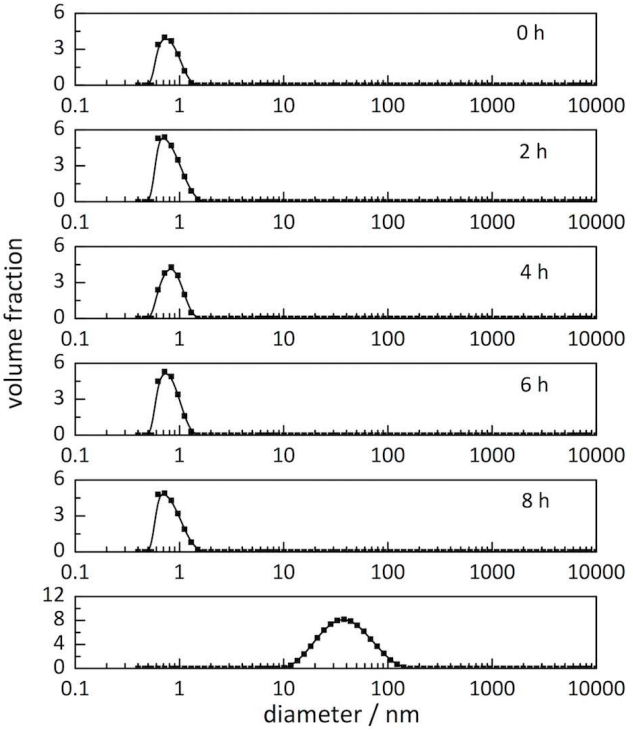


Fig 5. DLS data obtained during the enzymatic reaction as a function of time (top 5 frames) and after introduction of RNA (bottom).

In summary, the present work establishes an enzymatic control over formation of a cationic polyelectrolyte. Such concepts may prove useful in devising new strategies towards delivery applications as cationic charges are often employed for facilitating the cellular uptake of therapeutics due to their capacity to interact favorably with the negatively charged phospholipid components of the cell membrane.[27]

Notes and references

Department of Materials, ETH-Zürich, CH-8093, Switzerland. Fax: 41 44 633 1390; Tel: 41 44 633 6474; E-mail: anzar.khan@mat.ethz.ch

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