FAST ATRP OF *N*-ISOPROPYLACRYLAMIDE IN WATER AND ITS APPLICATION TO BIOCONJUGATES

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Introduction

Increasing utility of polymer-protein conjugates in medicine, biotechnology and nanotechnology has created the need for generating homogenous and defined biohybrids.[1-3] Well-defined polymer-protein conjugates can be prepared in one step by using modified proteins as initiating sites for atom transfer radical polymerization (ATRP). This technique has been shown to be advantageous not only for decreasing the number of synthetic steps but also offers the potential of controlling the site and the number of polymer chains conjugated to proteins.[4] Conjugation of protein to polymers which can respond to environmental stimuli is a great challenge.[5] Poly(*N*-isopropylacrylamide) (PNIPAAm) is a well-known thermo-responsive polymer and exhibits a lower critical solution temperature (LCST) of 32 °C in water.[6] Due to the possible denaturation proteins in organic solvents the conjugate should be synthesized in pure water. However experimental conditions to reach well-defined PNIPAAm by ATRP in water are to the best of our knowledge not yet reported.

Herein, we report a novel strategy to obtain PNIPAAm via ATRP in aqueous media with a functional initiator to allow protein conjugation. We detail the influence of the ratio $CuBr/CuBr_2$ or $CuCl/CuCl_2$ and of the choice of the ligand to access this polymer with an excellent control, without or with very tiny traces of irreversible termination even at very high conversion. Finally, the chemistry is applied to the conjugation of NIPAAm to horse spleen ferritin.

Experimental

Materials. NIPAAm (99%, Acros) was purified by two recrystallizations in a mixture of *n*-hexane and benzene. CuBr (98%, Aldrich) and CuCl (97%, Aldrich) were purified by stirring with acetic acid overnight. After filtration, they were washed with ethanol and ether and then dried in a vacuum oven. *N,N,N',N",N"*-pentamethyldiethylenetriamine (PMDETA; 99%, Aldrich) and 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA; 97%, Aldrich) were distilled before use. Tris(2-dimethylaminoethyl)amine (Me_6TREN) was prepared as described in the literature.[7] Water was obtained from a MilliQ PLUS (Millipore) apparatus.

Instrumentation. Polymers were characterized by size exclusion Chromatography (SEC) using a RI and UV(λ=270 nm) detection with 0.05 M solution of LiBr in 2-*N*-methylpyrrolidone (NMP) as eluent. PSS GRAM columns (300 mm x 8 mm, 7 µm): 10^3, 10^2 Å (PSS) were thermostated at 70 °C. ^{1}H-NMR spectra were recorded on a Bruker AC-250 spectrometer in $CDCl_3$ (residual peak δ = 7.26 ppm) at room temperature.

General Polymerization Procedure. For a typical polymerization NIPAAm (0.5 M) was dissolved with 2-bromo-isobutyric acid (BIBA) in 19 mL of pure water in presence of $CuBr/CuBr_2$ or $CuCl/CuCl_2$, respectively. The vial was capped with a rubber stopper to allow addition of the ligand and placed in an ice bath. In a second small flask 2 mL of aqueous ligand solution were prepared. Then both were deoxygenated by purging with nitrogen gas for 15 min. Afterwards 1 mL of ligand solution was withdrawn with a degassed syringe and placed in the polymerization flask to start the reaction. Samples were taken after pre-selected times and quenched with air.

Results and Discussion

Homopolymerization of N-isopropylacrylamide. NIPAAm was polymerized in the presence of bromo-2-isobutyric acid (BIBA). This initiator was chosen due to its high solubility in water and it has the advantage to carry a carboxylic group to allow protein modification by active ester chemistry. Another important parameter to succeed the polymerization is the temperature used. When polymerizations were carried out at room temperature with Cu(I) or with a high ratio Cu(I)/Cu(II), kinetics were extremely fast, typically less than a minute for full conversion. Due to the exothermic character of the propagation the temperature in the medium increased above the LCST of PNIPAAm (32 °C), leading to polymer collapse and loss of control. To avoid this problem, a rather low monomer concentration, typically $[M]_0$= 0.5 M and an ice bath was used to control the heat evolution. Under these conditions all polymerizations were successfully achieved even in the absence of Cu(II). Moreover, although the kinetics is fast, especially in the case of using Cu(I) alone or in presence of a high ratio Cu(I)/Cu(II), the GPC traces do not show a large amount of termination by coupling at very high conversion, which is the predominant termination reaction for acrylamide-based monomers. This property was useful in the case of Me_6TREN. Indeed, kinetic reproducibility was hard to achieve due to the high sensitivity of this ligand to oxygen which can drastically slow down the kinetics. Therefore the reactions were carried out at longer times than normally needed follow the kinetics, and then polymers with narrow molecular weight distribution at full conversion were obtained.

Table 1. Influence of the ligand for the ATRP of NIPAAm with CuCl in water at 4 °C[(a)]

Ligand	Time (min)	X_p (%)	M_n^{exp} (kg/mol)	PDI
PMDETA	95	>99	32	1.79
HMTETA	130	>99	29	1.94
Me_6TREN	76	>99	23	1.30

[(a)] $[M]_0$ = 0.5 M, $[M]_0/[BIBA]_0/[CuCl]_0/[CuCl_2]_0/[Ligand]_0$ = 100/1/1/0/1

A suitable choice of the ligand is crucial to reach a good control of NIPAAm polymerization. Inspection of the data given in Table 1 clearly indicates that *N,N,N',N",N"*-pentamethyldiethylenetriamine (PMDETA) and 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) are not really appropriate to obtain a polymer with a low polydispersity when $CuCl_2$ is not present in the media. In both cases, the GPC profiles showed a rather wide molecular weight distribution due to a long tailing in the range of the low molecular weight. This phenomenon can be explained by a slow initiation compared to the very fast propagation. Me_6TREN gives the best results due to the high activity of this ligand.

Table 2. Influence of the ratio Cu(I)/Cu(II) in the ATRP of NIPAAm in water at 4 °C with Me_6TREN as ligand[(a)]

Catalyst	$[Cu(I)]_0/[Cu(II)]_0$	Time (min)	X_p (%)	M_n^{exp} (kg/mol)	PDI
$CuCl/CuCl_2$	1/0	76	>99	23	1.30
$CuCl/CuCl_2$	0.6/0.4	85	>99	16	1.25
$CuCl/CuCl_2$	0.5/0.5	90	>99	16.5	1.20
$CuBr/CuBr_2$	1/0	60	>99	17	1.19
$CuBr/CuBr_2$	0.85/0.15	100	>99	18.5	1.08
$CuBr/CuBr_2$	0.7/0.3	115	>99	18	1.08
$CuBr/CuBr_2$	0.6/0.4	130	>99	19	1.09

[(a)] $[M]_0$ = 0.5 M, $[M]_0/[BIBA]_0/[catalyst]_0/[Ligand]_0$ = 100/1/1/1

The influence of the catalyst system was also investigated by comparing CuCl and CuBr based system at different ratio Cu(I)/Cu(II). Results are summarized in Table 2. In all cases CuBr provides a narrower distribution than CuCl, whatever the ratio Cu(I)/Cu(II) used. In the case of the bromide system, already without addition of $CuBr_2$ in the media, the polydispersity index is lower than 1.2. The addition of even a tiny amount of $CuBr_2$ makes it drop off to less than 1.1. This proves the excellent ability to polymerize NIPAAm with this system in a controlled fashion even if the kinetics is really fast. For the chloride based ATRP the molecular weight distribution is broader but decreased by raising the amount of $CuCl_2$. Moreover, like for CuBr the GPC traces look unimodal, symmetrical and no trace of termination by recombination can be seen even at full conversion.

Due to the fast character of the ATRP of NIPAAm in water, a kinetic study is rather difficult to perform when the ratio Cu(I)/Cu(II) is high. Therefore a ratio $[CuBr]_0/[CuBr_2]_0$ of 1/1 was selected to increase the reaction time. For a ratio $[NIPAAm]_0/[BIBA]_0$ of 50/1 and Me_6TREN used as ligand, the first-order time-conversion plot (Fig 1.) shows a linear relationship. This tendency indicates the absence of undesired side reactions. Moreover the molecular weight increases linearly with the conversion which proves the control of this reaction.

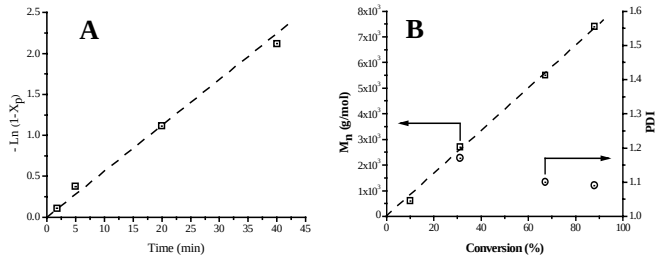

Figure 1. Kinetic of NIPAAm ATRP (0.5 M) in water at 4 °C with $[M]_0/[BIBA]_0/[CuBr]_0/[CuBr_2]_0/[Me_6TREN]_0$ = 50/1/0.5/0.5/1. (A) First-order time-conversion plot. (– –) Extrapolation. (B) Molecular weight and polydispersity index vs conversion. (– –) Extrapolation of the molecular weight.

To prove the versatility of this process, different chain lengths of PNIPAAm were synthesized. Figure 2 indicates that an increase of the ratio monomer/initiator leads (at a comparable conversion) to a linear increase of the molecular weight. The SEC traces display unimodal and narrow peaks. Moreover a large range of molecular weights from rather low (DP=30) to rather high (DP=400) can be obtained. In all cases the polydispersity index remains below 1.2 at full conversion, without any trace of termination. All these criteria indicate the controlled fashion of the ATRP of NIPAAm in water.

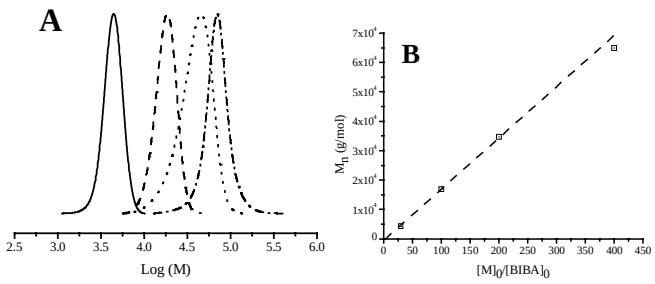

Figure 2. Influence of the ratio monomer initiator for the ATRP of NIPAAm (0.5 M) in water at 4 °C with $[BIBA]_0/[CuBr]_0/[CuBr_2]_0/[Me_6TREN]_0$ = 1/0.7/0.3/1. (A) Dependence of the molecular weight distribution for a ratio $[M]_0/[BIBA]_0$ = (——) 30, (– –) 100, (• • •) 200, (– • –) 400. (B) Evolution of M_n with the ratio $[M]_0/[BIBA]_0$

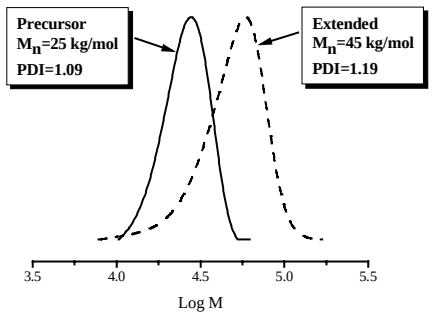

Figure 3. Molecular weight distribution for the chain extension of PNIPAAm by ATRP in water at 4 °C. $[M]_0$=0.5 M, $[M]_0/[PNIPAAm_{100}\text{-}Cl]_0$=300. (——) precursor, (– –) extension after 40% conversion

Chain Extension Experiments. To further demonstrate the livingness of the process a chain extension of a PNIPAAm precursor with NIPAAm was carried out. The initial block was obtained by using a ratio $[M]_0/[BIBA]_0/[CuCl]_0/[CuCl_2]_0/[Me_6TREN]_0$ of 120/1/1.6/0.4/2 with a NIPAAm concentration of 0.5 M. Then the block copolymer was synthesized by sequential addition after 38 min of a degassed aqueous solution of monomer (0.5 M) without purification of the macroinitiator. This strategy is only possible because the precursors were polymerized up to full conversion. Figure 3 depicts the evolution of the molecular weight distribution during the process. The GPC chromatograms show a distinct increase of the molecular weight. Moreover, as for the homopolymerization, even for a full conversion of the first block, throughout chain extension, there is no trace of a shoulder due to the termination by recombination. However a small tailing can be observed which can be due to a loss of terminal chloride of the precursor. Nevertheless such evidence combined with a low PDI suggests that the large majority of the PNIPAAm precursor retained the functionality and was available for subsequent chain extension.

Synthesis of bioconjugates. As a model protein particle for grafting-from studies, horse spleen ferritin was used. Ferritin possesses 24 chemically addressable primary amino end groups allow its conjugation with an initiator and short polymers.[8, 9] Ferritin was converted to an ATRP macroinitiator by attaching EBIB via active ester chemistry. A controlled growth of polymer chains from the protein shell is achieved by polymerization in presence of EBIB as sacrificial initiator. NIPAAm and also oligo(ethylene glycol) methacrylate (OEGMA) were polymerized directly in pure water by ATRP at low temperature, leading to polymer-grafted ferritin. The details are reported elsewhere.[10]

Conclusions

We have demonstrated that ATRP of NIPAAm can be carried out in water at low temperature. We also exhibited that the good choice of the ligand and the catalyst system is crucial to reach a good control due to the fast character of this polymerization. Under these conditions, the controlled/living characteristics were proven when BIBA, CuBr/CuBr and Me_6TREN were used. Besides, a large range of DP can be reached by this process. Moreover, even to full conversion the polymerization control is maintained. The living character of the generated PNIPAAm was confirmed by subsequent chain extension directly by addition of a second degassed monomer solution. Finally we demonstrate the advantage of this process to reach polymer-protein conjugate via a grafting from approach directly in water due to the carboxylic group present on the initiator which can be easily conjugate to protein via active ester chemistry.

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