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Improved Synthesis Strategy of Poly(amidoamine)s for Biomedical Applications: Catalysis by "Green" Biocompatible Earth Alkaline Metal Salts[a]

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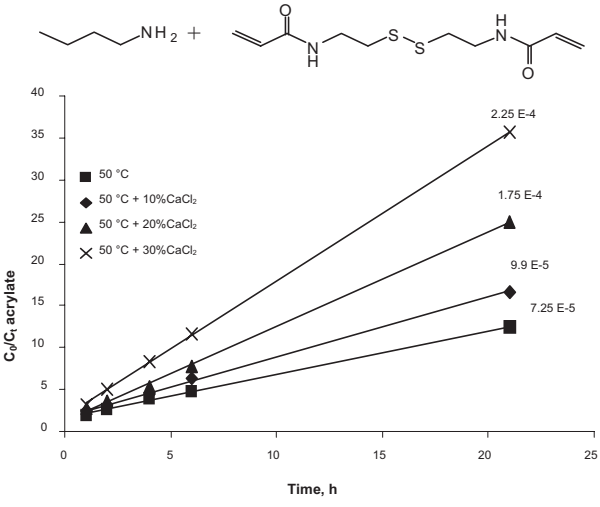
Poly(amidoamine)s (PAAs) have received significant attention due to their good biocompatibility and fast biodegradation profile which gives these polymers high potential in biomedical applications. Conventional synthesis of PAAs via aza-type Michael addition reaction of primary amines to *bis*-acrylamides often proceeds slowly and takes several days, which does not allow fast and extensive screening of PAA libraries for their bioactivity. Current investigation was dedicated to the development of catalytic synthesis procedures in order to decrease the polymerization times. The salts of several transition metals, as well as earth alkali metals were studied for their catalytic activity in the polymerization reaction. It was found that the salts of earth alkali metals showed the highest potential in the catalysis of polymerization, whereas the salts of transition metals showed either no effect or even resulted in slowing down the reaction. In particular, the addition of $CaCl_2$ to the reaction mixtures resulted in remarkable increase of the reaction rate as compared to the system without catalyst. PAAs synthesized by the conventional procedure and those obtained by using $CaCl_2$ as a catalyst showed no difference in physico-chemical properties as well as in biological activity. The novel synthetic method for PAAs, using catalysts based on earth alkali metals, represents an attractive alternative to currently applied methods. Characteristics of earth alkali metals such as low toxicity and good biocompatibility make them especially useful in the preparation of these polymers for biomedical applications.



Introduction

Poly(amidoamine)s (PAAs) have recently received significant attraction in the biomedical field due to their biodegradability, biocompatibility, and good performance as biomedical materials and polymer therapeutics.[1,2] PAAs can be obtained by Michael-type polyaddition of primary amines or *bis*-secondary amines to *bis*-acrylamides. Since the polymerization reaction is tolerable for a wide range of

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monomers, the structural variation that is possible in these polymers allows efficient adjustment of their properties for defined applications. For their application as drug and gene delivery vectors the hydrophilic/hydrophobic balance, the nature, and amount of different charged groups and the pK_a values of side groups in the polymer can be tuned to optimize cell adhesion properties, endosomolytic properties, payload incorporation, and biodegradability.[3–7]

Polymerization of PAAs is known to proceed as a second order reaction in protic solvents, whereas the highest reaction rate was found in water, which is often added to organic solvents as a mediator.[8,9] Since the reactivity of the different reactants is strongly dependent of their structure, the polymerization time could differ significantly, from several hours at room temperature to several weeks. Temperature elevation leads to higher polymerization rates.[10] However, it also increases the rates of side reactions (retro-Michael reaction and amide hydrolysis), which decreases the degree of polymerization of PAAs and alters the properties of the final product.[8] Therefore, only limited increase of the polymerization rate can be achieved by increase of the reaction temperature and typically the temperature during polymerization must not exceed 50 °C in order to avoid side reactions. Nevertheless, even at this higher temperature the polymerization often exceeds one week.[3–5,10]

A possible way to increase the polymerization rate and decrease the time of polymer synthesis is the use of an appropriate catalysts for the Michael addition reaction. It is known, that the conjugate addition of amino nucleophiles to α,β-unsaturated carbonyl compounds to form β-aminocarbonyl compounds can be catalyzed by Lewis acids and bases.[11] In particular, various metal catalyzed reactions have been studied in the presence of Pd compounds,[12] NaI/$CeCl_3 \cdot 7H_2O$,[13] $Bi(NO_3)$,[14] and $Yb(CN)_3$.[15] Xu et al.[16] screened several metal ions for aza-type Michael addition in aqueous solution and reported simpler and efficient metal catalysts such as $FeCl_3$, $SnCl_4$, and $CrCl_3$. The use of transition metals for synthesis of the materials for biological applications (such PAAs) raises, however, concerns about incomplete purification of the products from the catalyst. Especially in case of PAAs, which have rather high capacity for complexation of transition metal ions,[17–19] purification can represent an additional problem.

In the current paper we report a novel method for fast synthesis of PAAs using "green," biocompatible catalysts based on earth alkali metal salts, which show superior activity in Michael addition polymerization of PAAs over the salts of transition metals.

Experimental Part

The materials and methods used in this study are provided in Supporting Information. The polymerizations were carried out at 1:1 molar ratios between the reactants. For studies of the reaction kinetics, aliquots of the reaction mixture were taken after defined incubation times and analyzed by ^{1}H NMR (Supporting Information). After completion of the reaction the polymers were dialyzed against deionized water and freeze dried. The detailed protocols for the in vitro β-galactosidase gene transfection and cytotoxicity studies are provided in the Supporting Information.

Results and Discussion

In order to evaluate the potential of different metal ions for catalysis of the PAA polymerization, the reaction between methylene-*bis*-acrylamide (MBA) and butylamine (BA) was investigated as a representative model reaction (Table 1).

It can be seen that the reaction in water/methanol mixture proceeds significantly faster than in pure methanol, indicating that the presence of water in the reaction medium is important to facilitate the polyaddition reaction. Compared to the control mixture without any added salt, the addition of $CaCl_2$ resulted in a significant increase of the reaction rate, whereas all other salts showed either a moderate effect (Mg) or even retard the reaction (transition metals). In methanol, 80% conversion of the acrylamide groups was achieved already after 3 h, whereas in the control reaction this conversion was achieved only after 20 h reaction time. In 50% water/methanol mixture the polymerization reaction was accelerated approximately three times in the presence of $CaCl_2$ compared to the control reaction. Surprisingly and in contradiction to the findings of Xu et al.,[16] $FeCl_3$ decreases the reaction rate and the systems containing $FeCl_3$ showed the slowest kinetics among all metal salts under investigation.

The MBA used in the screening of the metal ion activity is a rather reactive acrylamide in Michael addition reactions. However, compared to MBA-based PAAs, the related PAAs based on *N*,*N*-cystamine-*bis*-acrylamide (CBA) received much higher attention in biomedical applications. This is

Table 1. Conversion of double bonds (%) in time during the MBA/BA polymerization catalyzed by different metal ions in methanol (A) and 1:1 v/v methanol/water (B). Conditions: Concentration of reactants 2 mol · L^{-1}. 1 equiv. MBA and 1 equiv. BA with 0.1 equiv. MCl_n at room temperature.

MCl_n	A			B	
	1 h	3 h	20 h	1 h	3 h
w/o	20	51	79	62	81
Mg	25	54	83	70	83
Ca	68	80	94	77	88
Zn	6	38	75	61	80
Cu(II)	12	42	79	61	78
Fe(III)	2	33	68	56	72

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due to the presence of the repetitive disulfide bonds in the polymer main chain, which enables relatively fast biodegradation of the biomaterials in the reductive intracellular environment. Such properties make CBA based PAAs very attractive in delivery of drugs, proteins,[20] and nucleic acids.[1,3–5,21] The reactivity of CBA is rather low and the conventional synthesis of CBA based PAAs typically proceeds during 7–10 d at 50 °C,[5,7] which does not allow fast extensive chemical screening of related PAAs. Therefore, it was of considerable interest to further investigate the catalytic activity of $CaCl_2$ on the reaction of BA with CBA (Figure 1).

The polymerization proceeds as a bimolecular reaction with second order kinetics.[8] Therefore, in Figure 1 the kinetics of the reaction are expressed in the ratio between actual and initial concentrations of the reacting species (acrylamide groups) versus time. Using these coordinates linearity of the data should be obtained. It can be seen that the reaction under the conventional conditions (50 °C) proceeds relatively slow. An increase of the temperature to 70 °C led to some increase of the reaction rate, reaching 90% of conversion after 14 h of incubation (Figure 1a). However, the reaction rate is still rather low considering that in polyaddition reactions significantly higher conversions should be achieved (typically approx. 99%) in order to achieve high molecular weights of the polymers. Using the kinetic constants obtained from the graph, this degree of conversion could be reached after 160 h or 7 d. Further increase of the temperature to 90 °C led to further acceleration of the reaction during first few hours. However, in the later stages (after approx. 5 h) the kinetic curves started to lose linearity, indicating an increasing influence of concurrent reactions (hydrolysis of amide bonds). This conclusion is supported by comparison of the physicochemical properties of the polymerizates obtained at 50 and 90 °C. For polymerizates incubated at 50 °C in water/methanol 1:2 v/v mixture with conversions below 80% clear solutions are formed even if cooled to room temperature. For conversions between 80 and 90% a gradual increase of the critical solution temperature could be monitored and for conversions above 90% phase separation occurs even at the reaction temperature (50 °C). In case of polymerizates synthesized at 90 °C no phase separation was monitored even after cooling the reaction mixture to room temperature. Thus, only limited acceleration of the polymerization rate of PAAs by temperature increase can be achieved due to significant increase of side reactions at elevated temperatures.

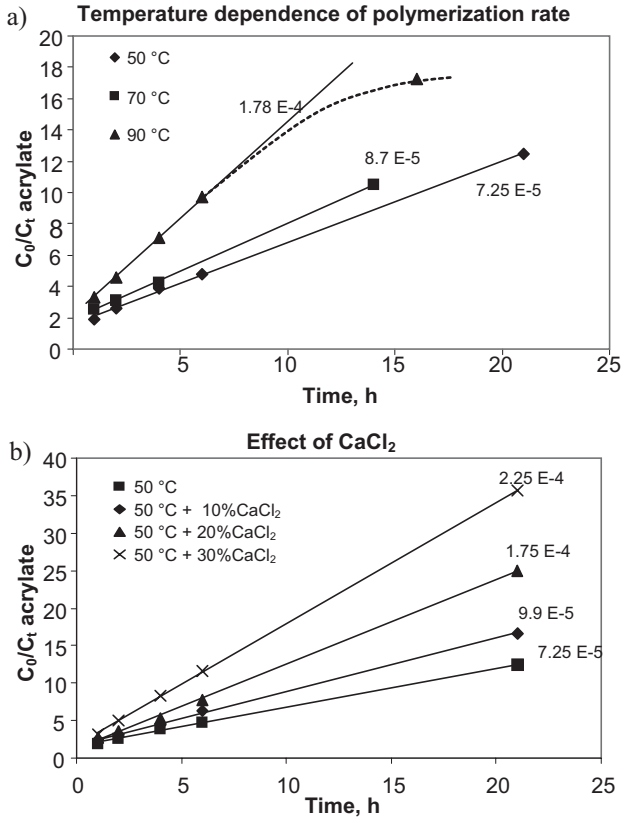

Figure 1. Polymerization kinetics of BA with CBA at different temperatures (a) and different concentrations of $CaCl_2$ (b). C_o and C_t are the initial and actual concentration of acrylate groups in the reaction mixture, respectively. The numbers near the kinetic curves represent the calculated kinetic constants in $M^{-1} \cdot s^{-1}$.

Addition of $CaCl_2$ to the reaction mixture significantly increases the reaction rate (Figure 1b). Even at 50 °C the reaction proceeds faster than at 90 °C without catalyst. The reaction rate gradually increases with increase of the amount of catalyst (Figure 1b). In presence of $CaCl_2$ the desired conversions of 98–99% could be achieved during 1 d of synthesis in comparison to more than 1 week required without catalyst, which represents a significant improvement of current synthetic procedures of PAAs. No catalytic degradation of the polymer by hydrolysis of the amide functions in the presence of Ca ions could be detected by NMR.

The molecular parameters of PAAs composed of CBA and 4-amino-1-butanol (ABOL) synthesized in presence of $CaCl_2$ were evaluated. For this purpose the aliquots of the reaction mixture were taken at defined time points and the reaction was terminated with *tert*-BA and the dialyzed samples were analyzed by NMR. It was assumed that the terminated polymer exists of polymer chains containing statistically one *tert*-butyl end group. This allows the estimation of number-average molecular weight by NMR based on the peak area of this endgroup relative to the areas of two methylene peaks from the butanolic side chain (see SI-Figure 1). It can be seen in Figure 2a that the molecular weight of the resulting polymer quickly increases to *ca.* 10 kDa during the first 10 h of polymerization at 70 °C in presence of 10 mol-% of $CaCl_2$, followed by a more gradual

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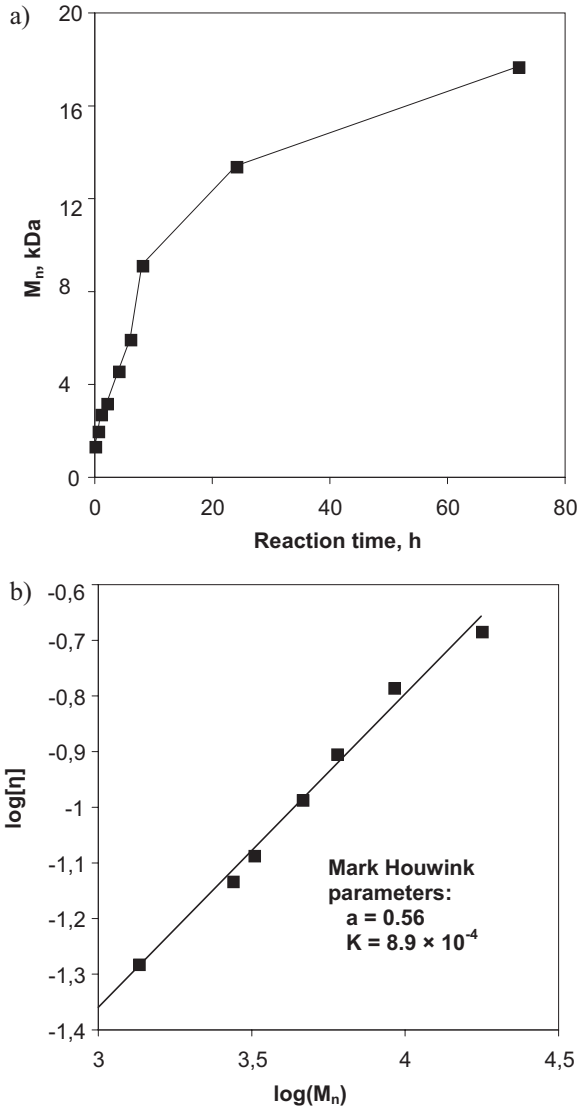

Figure 2. Dependence of the molecular weight of CBA/ABOL polymers on the reaction time (a) and determination of Mark–Houwink parameters of resulting PAAs (b). Reaction conditions: 70 °C, 10 mol-% $CaCl_2$.

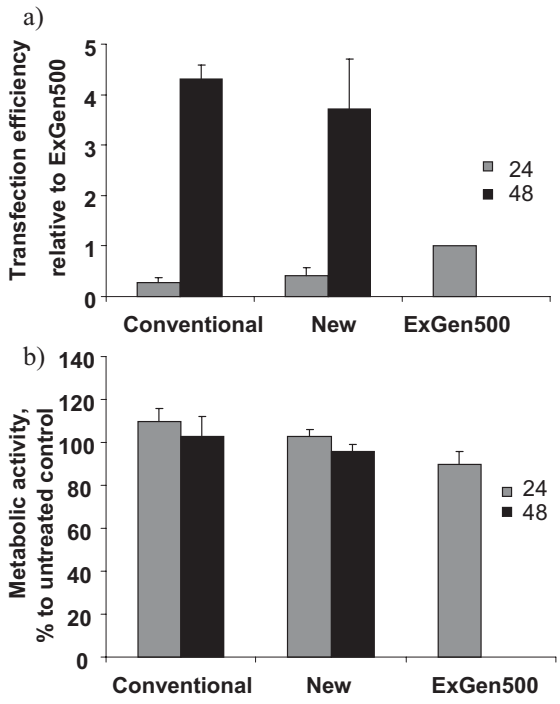

Figure 3. Efficiencies of β-galactosidase transfection (a) and cell viabilities (b) of polyplexes prepared at 24 and 48 polymer/DNA weight ratios in COS-7 cells, using CBA/ABOL polymers synthesized in the presence of $CaCl_2$ catalyst (new) or without $CaCl_2$ (conventional). Transfection efficiencies represent the values relative to transfection efficiencies of the commercial agent ExGen 500.

increase of *ca.* 18 kDa after 75 h of reaction time. The intrinsic viscosities of all polymer samples showed an excellent linearity with the estimated molecular weights in a double logarithmic plot, allowing also the evaluation of the Mark–Houwink parameters for the CBA/ABOL polymer (Figure 2b). Since both increase in temperature and $CaCl_2$ concentration led to acceleration of the polymerization and increase of temperature most probably have the largest effect on the occurrence of possible side reaction, like hydrolysis, it was of interest to investigate whether a decrease in reaction temperature from 70 to 50 °C and an increase of the amount of $CaCl_2$ in the reaction mixture to 20 mol-% would further optimize the polymerization reaction. The molecular weights of PAAs synthesized at 50 °C in the conventional way and in the presence of 20% of $CaCl_2$ were 17 and 18 kDa, respectively, calculated from the determined Mark–Houwink parameters (Figure 2b). Thus, only the temperatures above 70 °C could significantly affect the rate of amide hydrolysis and consequently decrease the molecular weights.

Since PAAs are of particular interest for their potential biological applications, the possible effect of the use of the Ca catalyst on biological activities of the products was evaluated. For this purpose the polymer of CBA and ABOL was synthesized both in the absence and presence of $CaCl_2$. The CBA/ABOL polymer was previously found to be a very promising transfection agent, which shows superior efficiency in cell transfection over commercially available transfection agents (e.g., linear PEI) without any toxicity in vitro.[22] Two batches of this polymers, one synthesized at 50 °C in the conventional way (10 d reaction time) and one synthesized in the presence of 20 mol-% of $CaCl_2$ (30 h reaction time) were compared. It is shown that the both samples showed similar transfection efficiencies in a whole range of mixing ratios between DNA and the polymer (Figure 3). Moreover, no difference in cell toxicity was observed between the two polymers as both batches showed around 100% cell viability.

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Conclusion

A pronounced catalytic activity of $CaCl_2$ on the Michael addition polymerization of PAAs was demonstrated. The reaction rate was proportional to the concentration of catalyst in the reaction mixture. Addition of this catalyst to an equimolar mixture of *bis*-acrylamide and primary amine resulted in up to 10-fold reduction of synthesis time as compared to systems without added catalyst. $CaCl_2$ represents an attractive, biocompatible, and non-toxic alternative to transition metal catalysts, which is especially important in the synthesis of biomedical polymers. The polymers resulting from catalytic synthesis have similar physicochemical characteristics and biological activities as the polymers synthesized in the conventional way. This novel synthetic approach represents a significant improvement of currently applied routes for synthesis of PAAs and provides better possibilities for extensive fast chemical screening of different PAA structures for biological applications.

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