Towards DNA sensing polymers: interaction between acrylamide/3-(N, N-dimethylaminopropyl)-acrylamide and DNA phage λ at various N/P ratios

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The present study strongly relates to ongoing research on the development of cationic polymers which are of great interest due to their enormous potential for biomedical applications, especially as non-viral vectors for gene therapy, antimicrobial agents and active components in DNA sensing devices. The current paper demonstrates that a functional group approach can be successfully realized in a free-radical copolymerization process for preparing cationic copolymers with a desired contribution of amine groups, which being protonated in water, provide electrostatic interactions between a polycation and DNA. Three replicas of the cationic copolymer, acrylamide/3-(N, N-dimethylaminopropyl)-acrylamide (AADMAPA), were synthesized using this strategy. The values of number average molecular mass and polydispersity index, being similar for the three replicas, averaged to 24000 ± 2000 g/mol and 1.5 ± 0.1 , respectively. The copolymer composition, examined by $^1\text{H-NMR}$ (D_2O), was corresponded to the molar ration of initial monomers. The dynamic light scattering studies and zeta potential measurements confirmed that in water positively charged AADMAPA /DNA polyplexes are formed at $\text{N/P} > 2.2$: bimodal distributions of particles, whose diameters average 70 and 700 nm, were observed. Zeta potential measurements indicated that at $\text{N/P} \approx 2.2$ the charge-neutralization point is observed. The Atomic Force Microscopic study showed that positively charged AADMAPA /DNA polyplex is an axially symmetric particle.

Introduction

Why does a novel biomedical high-tech area pay special attention to cationic polymers? The unique physico-chemical and biochemical properties of cationic polymers give answer to this question. Due to the presence of positively charged groups cationic polymers can interact with DNA's negatively charged phosphates groups by means of electrostatic attraction, resulting in the formation of compact, spherical-like complexes (polyplexes). These polymer systems can be employed in a number of various biomedical applications such as DNA sensing, controlled delivery of therapeutic agents or non-viral vectors for gene therapy etc.¹⁻¹⁶ Thereby, a large number of synthetic cationic polymers have been synthesized and characterized; the most widely used and studied cationic polymers include poly(ethyleneimine) (PEI) poly(lysine) (PLL), poly(2-(N,N-dimethylamino)ethyl methacrylate] (PDMAEMA); poly(vinyl amine) (PVA).¹⁷⁻²¹

The majority of the cationic polymers bears an amine function that can be protonated; the relative number of the protonable amines differs for each polymer. The resultant characteristics (size and size distribution, transfection efficiency, stability, toxicity etc.) of polycation/DNA polyplexes depend on many parameters including chemical structure and the molar mass of an initial polycation, the DNA solution concentration, polymer to DNA molar ratio (N/P ratio), pH etc.^{22, 23} Moreover, the average size and the surface charge of the formed polyplexes as well as initial polymer architecture have great impact on their transfection efficiency.^{24, 25} It was also demonstrated that the greater the distance between the charged groups in a polycation, the higher its ability to safely and efficiently deliver DNA into the cell.²⁶ On the other hand, it is clear that with higher amount of positively charged groups DNA holds stronger by cationic copolymer. Therefore, for the use of cationic polymers in the gene delivery process as well as in DNA sensing applications, the development of synthetic strategy that may permit engineering cheap polycationes with desired DNA holding properties is one of the important challenges

of modern biomedicine. The above conclusions motivated us to realize a functional group approach in combination with free-radical copolymerization for preparing a new cationic copolymer with a controlled contribution of amine groups. This synthetic strategy (**Figure 1**) permitted us to prepare a water soluble bioactive cationic copolymer - acrylamide/3-(N, N-dimethylaminopropyl)-acrylamide (AADMAPA), and showed that it is able to form complexes with DNA.^{27, 28} In relation to this result two fundamental questions arise: (1) does this synthetic procedure permit one to reproducibly prepare AADMAPA with a desired composition? and (2) what is the correlation between N/P values and characteristics (size and charge) of AADMAPA/DNA complexes formed in water? Getting answers on these questions was a main subject of our research that we present and discuss in this article.

Here we demonstrate on the example of three synthetic replicas that the developed preparation of AADMAPA is a well reproducible synthetic method. We also present and discuss the study of the complexation behaviour of AADMAPA with DNA phage λ in water at various N/P ratios. In particular, the article reports the physico-chemical properties of the AADMAPA and AADMAPA /DNA polyplexes studies using different characterisation methods such as Size Exclusion Chromatography (SEC), dynamic light scattering (DLC) and atomic force microscopy (AFM).

Experimental

Synthesis of the bioactive cationic copolymer: acrylamide/3-(N, N-dimethylaminopropyl)-acrylamide (AADMAPA)

N,N,N',N'-Tetramethylethylenediamine (TEMED) (12 μ l, 0.08 mmol) was added to the mixture of acrylamide (AA) and N-(3-dimethylaminopropyl)-acrylamide (DMAPA) preliminary dissolved in water (9.5 ml); the molar ratio AA:DMAPA was 0.85:0.15. The mixture was heated to 50 °C under stirring and ammonium persulfate (0.004 g, 0.0175 mmol) dissolved in the 0.5 ml of water was added to the reaction mixture. Once the mixture was stirred for one hour at 50 °C, it was cooled down to the room temperature and dialyzed against low molecular weight (up to 10 kDa) fractions. The final fraction contained water solution of AADMAPA. Water was then removed by freeze-drying. Three replicas of this synthetic protocol have been performed (see **Table 1**). The copolymer was generated in high yields in the range from 71 % to 76 %. The chemical structure of the copolymer is shown in **Figure 2**.

NMR spectra

Proton NMR spectra were recorded in D₂O using a Bruker Model ARX (300 MHz) spectrometer. ¹H-NMR data were additionally confirmed by copolymer's IR spectra collected on a Bruker Tensor 37 in the range 3,500 - 1,000 cm⁻¹ (**Table 2**, **Figure 1 SI** and **Figure 2 SI**, ESI).

Size exclusion chromatography

Size Exclusive Chromatography(SEC) was performed on SEC system (Waters Chromatography Division) using Hysdrogel (120 A and 250 A) column, mobile phase with 5% NaH₂PO₄ and 4.3% CH₃CN, pH 4, flow rate 0.8 ml/min, T = 36 °C, dRI M410 and UV M490 detectors, polystyrene calibration.

Preparation of AADMAPA/ λ -phage DNA complexes

All experiments were conducted with λ -phage DNA (48.5 kbp, Fermentas). Its AFM image is shown in **Figure 3 SI A** (ESI). To prepare AADMAPA/ λ -phage DNA complexes with different N/P ratios the estimated amount of the aqueous DNA solution with the concentration of 300 μ g/ml (see **Table 1 SI**, ESI) was added to the copolymer water solution (1 ml, 100 μ g/ml). After addition of λ -phage DNA, the mixture was extensively shaken. The polyplexes were then incubated for 30 min at room temperature.

Particle size and zeta-potential measurements

The particles size and the zeta-potential measurements were performed at 25°C using DLS (Zetasizer Nano ZS) and Dispersion Technology Software V.5.10 (Malvern Instruments Limited) to analyze the data. Each sample was measured 8 times with 6 single runs per measurement. All measurements were performed in water at pH 6.5.

Atomic force microscopy

Samples for AFM were prepared on the surface of freshly cleaved mica. A 2.5 µl droplet of the solution (polyplexes or initial DNA) was deposited onto mica and dried at room temperature for 24 h. AFM investigation was performed in air at room temperature using scanning probe microscope FemtoScan (Advanced Technologies Center). MikroMasch cantilevers of the 15 series were used in the tapping mode. Their average resonance frequency was 325 kHz, and the tip radius was about 10 nm. AFM images were processed and analysed with the help of FemtoScan Online software.

Results and discussion

Preparation of the cationic polymer AADMAPA

As was mentioned in the Introduction, the very important feature of synthetic cationic polymers is that they can be synthesized to match required properties for the optimization of the gene delivery process or to be used in other applications. The synthetic strategy, used in our research, fully justifies this approach. Recently, our preliminary data demonstrated that through controlled free radical copolymerization, using monomers such as AA and DMAPA, water soluble cationic acrylamide-based copolymer with a desired amount of functional groups may be prepared.²⁷ In the context of our synthetic approach for engineering bioactive copolymers, a fair question to ask is how reproducible the developed of synthetic method must be in order to be useful? One way to answer the question of reproducibility is to get reproducible values of the yield of a product and its composition. With this aim three replicas of AADMAPA were prepared by controlled free radical copolymerization that uses the red-ox system of ammonium persulfate – TEMED as a source of free radicals. All replicas of AADMAPA were characterized by SEC, ¹H-NMR and IR spectra. The results are summarized in **Table 1** and **2** (see the Experimental part). As one may see from the data presented in **Table 1**, three replicas of AADMAPA were prepared in high yields in the range 71 – 76 %. The values of number average molecular mass and polydispersity index, being similar for the three replicas, averaged 24000±2000 g/mol and 1.5± 0.1, respectively. It should be noted that ¹H-NMR and IR spectra of the replicas showed characteristic signals for the polymer backbone and functional groups (**Table 2**). Three samples of AADMAPA have the same composition: the molar ratio of AA to DMAPA was determined as 0.85 to 0.15. This was justified by reference to integral intensities of the ¹H-NMR signals corresponding to the protons of CH and N(CH₃)₂ groups. We emphasize that the copolymer composition is corresponded to the molar ration of initial monomers; this result is of special importance for us because it demonstrates that our synthetic approach allows engineering cationic polymers with the controlled amount of amine-based functional groups. This outcome, coupled with the reproducible values of both the yield of the cationic copolymer and its composition, reveals that the developed synthetic strategy is useful for engineering polycations with the desired amount of functional groups.

The complexation behavior of AADMAPA with DNA phage λ

Here it should be noted that the study of the AADMAPA/DNA polyplex formation is crucial to further enhance the rational design and functionality of the polymer and as a consequence the applications based on it. Taking into account that positively charged complexes polycation/DNA may be used for gene transfection, the main attention was given to the definition of the N/P values at which stable positively charged AADMAPA/DNA phage λ complexes can be formed. For this purpose we added small volumes of saturated λ-phage DNA solution (20-200 µl) to 1000 µl of the AADMAPA water solution. The used procedure permitted us to carry out the study the AADMAPA/λ-phage DNA condensation at almost constant concentration of AADMAPA. Both **Figure 3A** and **Table 2 SI** (ESI) clearly show that when N/P values decreased from 10 to 3.3, zeta-potential is positive and practically constant; it is equal to 20-21 eV, indicating the formation of

positively charged AADMAPA/DNA complexes, the size distribution of which is bimodal with peaks at ≈ 70 and ≈ 700 nm (**Figure 3B**). The hydrodynamic radius of the pure λ -phage DNA is about 130 nm, and it slightly depends on DNA concentration in the range from 25 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ (**Figure 4 SI**, ESI). Therefore the complexation of λ -phage DNA with AADMAPA leads to the DNA compaction. In this context, we assumed that the first peak could be assigned to the complexes of AADMAPA with the single λ -phage DNA molecule. Such nanostructures tend to aggregate into the higher order complexes resulting in the second peak observed at 700 nm. Here, it should be noted that according to M.A.Mintzer et al., polyplexes with 75 nm and 87 nm diameters demonstrated the highest transfection efficiency in comparison with complexes of other sizes.¹⁷

When N/P values decreased from 3.3 to 1, zeta-potential decreases dramatically: the effective charge of the resulting complexes changing from positive to negative values. The results obtained from zeta-potential measurements indicate that nearly all the DNA chains are complexed with AADMAPA when N/P ratio reaches ≈ 2.2 . This is in good correlation with reported data which repeatedly showed that the DNA molecules are completely complexed with polycations containing amine groups at N/P (≈ 2 -3).^{29, 30} As one may see from **Figure 3B**, the bimodal size distribution of the AADMAPA/DNA nanocomplexes is observed in the range $1.5 \leq \text{N/P} \leq 10$, when $\text{N/P} < 1.5$ the size distributions of the particles demonstrates three distinct peaks at ≈ 20 nm, ≈ 100 nm and ≈ 700 nm. A new particle population with a very low average diameter may be a product of the polyplexes decomposition.

Polyplex morphology

The polyplexes morphology was investigated by AFM, the results are presented in **Figure 4**. AFM investigations revealed that a smooth film was formed on the surface of mica after drying the solution of the initial copolymer (**Figure 3 SI B**, ESI). The mean square roughness of the film is about 0.4 nm. Any signs of the copolymer self-aggregation were not observed.

DLS and zeta-potential measurements showed that stable positive charged polyplexes are formed at N/P ratio in the range from 3.3 to 10. In this context, the AFM study was carried out for samples with N/P ratio of 5. It revealed the axial symmetry of the polyplexes and their bimodal size distribution (**Figure 4A**). It can be assumed that the large particles with the diameter of 500 – 1000 nm and the height of 30 – 80 nm are agglomerates of small particles with the diameter of 50 – 300 nm and the height of 1 – 25 nm, which is in good correlation with the DLS data. The latter may consist of a single DNA molecule surrounded by the positively charged copolymer. Most likely, the particles have a spherical shape in the solution, which is flattened due to the adsorption on mica. The same effect was observed when polyplexes were investigated by AFM in liquid environment.³¹ It has been reported previously that long DNA chains favors the formation of spherical nanoparticles, which is consistent with our results for the λ -phage DNA.³² For the comparison of AFM and DLS data, the diameter of spheres with a volume equal to the volume of particles found on mica was calculated. The relatively small and large particles have the diameter of 30 ± 20 nm and 260 ± 90 nm, respectively. These values are 2 – 3 times lower than the polyplex sizes measured by DLS. As the polyplex sizes measured by DLS and AFM in liquid phase are in the same range, such inconsistency may be explained by the decreasing of the polyplex volume upon drying on a mica surface.³³

In order to explain the appearance of the third peak in the size distribution, the samples with N/P ratio of 1.25 were studied by AFM. The polyplexes obtained at $\text{N/P} = 1.25$ have a more complex structure (**Figure 4B**). They consist of a dense core with the diameter of 300 – 1000 nm, and the height of 15 – 45 nm. The core is surrounded by a crown comprising of filamentous fragments, with the height of several nanometers, which could be attributed to the not fully complexed DNA chains. The diameter of the equivalent spheres with the same volume as the volume of the polyplexes dense core is 200 ± 60 nm. Thus, at low N/P the complexes lost their compact spherical shape. A new population of particles with sizes of about 20 nm most probably corresponds to DNA strands, partly bound with the molecules of the copolymer.

Conclusions

It was shown that in the range of $1 \leq N/P \leq 10$ the cationic acrylamide/3-(N, N-dimethylaminopropyl)-acrylamide copolymer forms complexes with DNA; charge neutralization point is corresponded to N/P as 2.2. The compact positively charged spherical polyplexes forming at the excess of the copolymer have prospects as intelligent nanocarriers for drug and gene delivery.

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