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Article

Fluorescent nanosized PAMAM dendrimers: One-step formation of a bright blue fluorophore on terminal groups and its optical properties

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Abstract. ***Background and Objectives*:** Polyamidoamine dendrimers (PAMAM) are nanoscale monodisperse compounds with a multifunctional terminal surface. Structural features of PAMAM, such as a nanosize of high homogeneity, highly developed terminal surface and cavities in the structure open up wide possibilities for their application. The most promising use of PAMAM is for biomedical purposes, in particular for the targeted drug delivery (for example, anticancer drugs). The interaction of PAMAM with target cells can be assessed using fluorescent imaging. This suggests the preliminary modification of PAMAM with various fluorescent molecules or the development of approaches to increase the intrinsic fluorescence of PAMAM. ***Materials and Methods*:** In this paper, we will consider a one-step modification of PAMAM based on the double cyclization reaction of PAMAM terminal groups and citric acid. Two approaches are chosen for modification: hydrothermal and boiling methods. The methods of optical spectroscopy and dynamic light scattering will be used as the main research tools. The methods used make it possible to determine the efficiency of fluorophore formation under given conditions. ***Results*:** In this work, we have proposed and implemented a one-step modification of PAMAM with a bright blue fluorophore (1,2,3,5-tetrahydro-5-oxo-imidazo[1,2-a] pyridine-7-carboxylic acid, IPCA), which is formed by a double cyclization reaction between citric acid and terminal ethylenediamine fragments of PAMAM. It has been shown that as a result of modification the hydrodynamic diameter of PAMAM does not change, the fluorescence intensity increases significantly (the quantum yield increases from < 1 to 28%), ζ-potential changes from 42 ± 5 to -24 ± 4 mV. ***Conclusion*:** Reaction of PAMAM and citric acid leads to the appearance of bright-blue fluorescence, which is significantly higher than the intrinsic fluorescence of PAMAM. A combination of bright fluorescence and a multifunctional terminal surface make it possible to further use the obtained structures for biovisualization
Keywords: PAMAM, citric acid, fluorescent nanostructures, one-step modification

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Научная статья
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Флуоресцентные наноразмерные дендримеры ПАМАМ: одностадийное образование ярко-синего флуорофора на концевых группах и его оптические свойства

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Аннотация. Полиамидоаминовые дендримеры (ПАМАМ) представляют собой наноразмерные монодисперсные соединения с многофункциональной концевой поверхностью. Структурные особенности ПАМАМ, такие как наноразмерность высокой однородности, сильно развитая концевая поверхность и полости в структуре, открывают широкие возможности для их применения. Наиболее интересным является использование ПАМАМ в биомедицинских целях, в частности для адресной доставки лекарственных препаратов (например, противоопухолевых). Взаимодействие ПАМАМ с клетками-мишенями можно оценить с помощью флуоресцентной визуализации. Это предполагает предварительную модификацию ПАМАМ различными флуоресцентными молекулами или разработку подходов для увеличения собственной флуоресценции ПАМАМ. В данной работе предложена и реализована одностадийная модификация ПАМАМ ярко-синим флуорофором (1,2,3,5-тетрагидро-5-оксо-имидазо[1,2-a] пиридин-7-карбоновая кислота, ИПКК), который образуется в результате реакции двойной циклизации концевых групп ПАМАМ и лимонной кислоты. Показано, что в результате модификации гидродинамический радиус ПАМАМ не изменяется, значительно увеличивается интенсивность флуоресценции (квантовый выход увеличивается с $<$1 до 28 %), ζ-потенциал изменяется с 42 ± 5 до -24 ± 4 мВ.
Ключевые слова: ПАМАМ, лимонная кислота, флуоресцентные наноструктуры, одностадийная модификация

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1. Introduction

Polyamidoamine dendrimers (PAMAM) are hyperbranched polymers with molecular uniformity, defined size and shape characteristics and a multifunctional terminal surface. These nanoscale polymers consist of an ethylenediamine core, a repetitive branching amidoamine internal structure and a primary amine terminal surface. Dendrimers are "grown" off a central core in an iterative manufacturing process, with each subsequent step representing a new "generation" of dendrimer. Increasing dendrimer generation produces larger molecular diameters and molecular weight, as well as an enhancement of reactive surface areas, which are functional keys to use of PAMAM dendrimers. PAMAM of low generations exhibit almost linear geometry, later generations show more globular-like shapes (G4.0 and above) [1]. The cavities that are intrinsically present in the globular shapes of PAMAM make them suitable agents for encapsulating and adsorbing biomolecules [2–4], and for use as prodrugs [5]. The ability of PAMAM to cross the blood-brain barrier makes their application for targeted antitumor therapy very promising [2, 6]. Perspectives of biomedical applications boost the development of functional PAMAM derivatives, that combines intrinsic PAMAM carrier properties and functionality of modifiers: targeting ligands that provide address delivery [3, 7–10]; fluorescent molecules [7, 9] and/or contrast agents [3, 11] to simplify visualization of the target area by fluorescence and/or magnetic resonance contrast. As a rule, approaches to PAMAM surface modification are multi-stage and labor-intensive, which necessitates the development of simpler methods.

PAMAM dendrimers have very low intrinsic fluorescence (quantum yield $< 1\%$), which limits their use for biomedical imaging, since the contrast between PAMAM fluorescence and cell autofluorescence is minimal [12–14]. Despite the existing approaches to increasing the intrinsic fluorescence of PAMAM (for example, oxidative treatment [14], acid-base transformation reactions [15] and solvent-induced PAMAM aggregation [16]), surface modification with fluorescent molecules is more effective [7–9].

In this work, we report the possibility to use terminal fragments of ethylenediamine of PAMAM G4.0 for reaction with citric acid (CA) to obtain PAMAM derivative (PAMAM–CA) with

bright blue fluorescence. This approach is based on the previously well described interaction of CA and amine -containing agents, particularly, 1,2-ethylenediamine (EDA), which produces a bright blue fluorophore (1,2,3,5-tetrahydro-5-oxo-imidazo[1,2-a] pyridine-7-carboxylic acid, IPCA) as a result of the double cyclization reaction (Fig. 1). This reaction has already been considered for EDA and its low molecular weight derivatives [17–19], as well as for surface modification of silica nanoparticles with terminal amino groups [20], but has never been used to modify PAMAM. Thus, we describe two options for a simple one-step modification of PAMAM G4.0 with a bright blue fluorophore (IPCA) as a result of the reaction between the terminal fragments of PAMAM and CA; and study the properties of the modified PAMAM. The use of boiling under reflux at atmospheric pressure and hydrothermal treatment made it possible to obtain the modified fluorescent PAMAM with identical characteristics. This is promising for creating drug delivery systems with the possibility of fluorescent visualization.

2. Experimental section

2.1. Modification PAMAM with a bright blue fluorophore (IPCA)

Two approaches of treatment of PAMAM and CA aqueous solutions were chosen for the PAMAM modification: boiling under reflux at atmospheric pressure and hydrothermal treatment in a closed volume (Fig. 2).

To modify PAMAM, a 10 wt.% solution of PAMAM G 4.0 in methanol was mixed with an aqueous solution of CA. The molar ratio of the reagents was 1 : 64 and the resulting concentrations were $7 \cdot 10^{-5}$ and $4.5 \cdot 10^{-3}$ mol/l for PAMAM and CA, respectively. For the boiling method, 10 ml of the resulting solution was transferred to a round

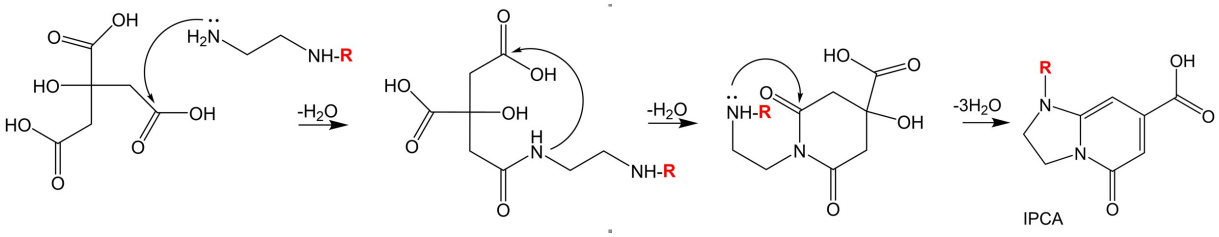

Fig. 1. Scheme of the formation mechanism of IPCA molecular fluorophore from CA and EDA-derived compound [18–20]

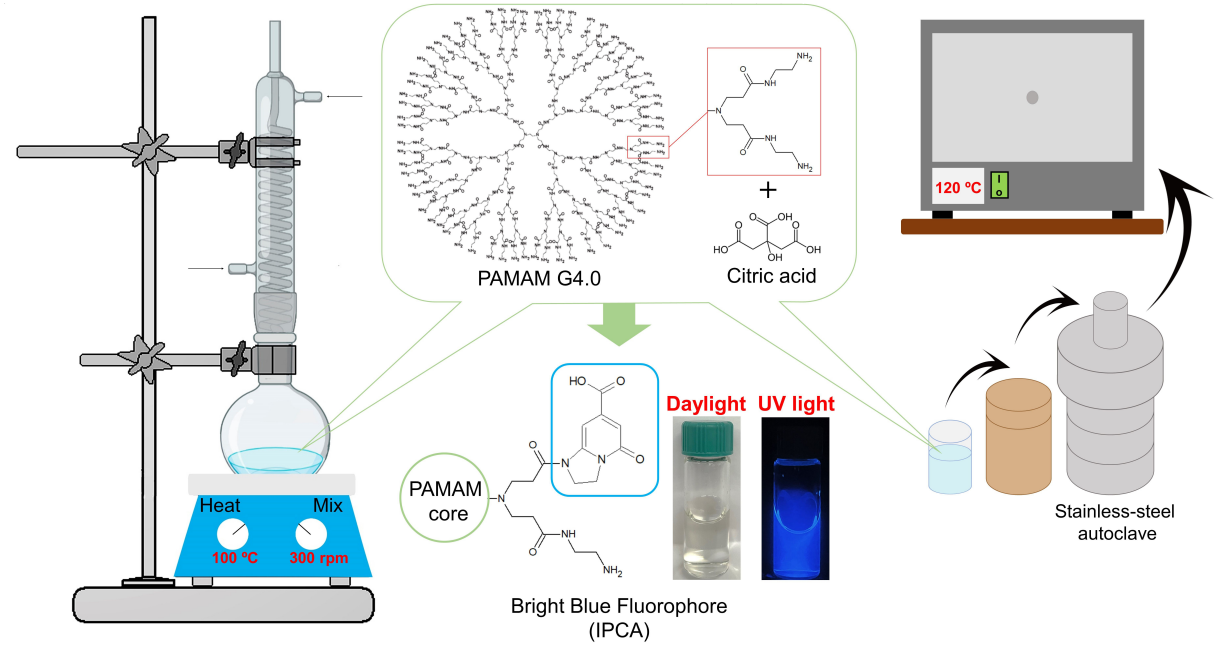

Fig. 2. Scheme of PAMAM modification with a bright blue fluorophore: Boiling under reflux (left) and hydrothermal treatment (right) (color online)

bottom flask connected to a reflux condenser and heated to 100°C for 4 hours (Fig. 2, left; boiling at atmospheric pressure). For the hydrothermal treatment, 4 ml of the resulting solution was transferred into a glass beaker inside a teflon-lined stainless-steel autoclave and heated at 120°C for 4 hours (Fig. 2, right; hydrothermal treatment). As a result of hydrothermal treatment of aqueous solutions, an increased pressure is created ($\sim$200 kPa a temperature of 120°C).

2.2. Characterization of samples

Absorption spectra were recorded by Shimadzu UV-1800 (Shimadzu Corporation, Kyoto, Japan). Emission and excitation spectra were obtained by a Cary Eclipse spectrometer (Agilent Technologies, Mulgrave, Victoria, Australia). The average diameter and zeta-potential (ζ-potential) of samples were analyzed with a Zetasizer Ultra (Malvern Panalytical, Worcestershire, UK).

2.3. Quantum yield calculation

Calculation of the quantum yield (QY) allows one to estimate the efficiency of emission. The relative QY of samples was calculated using quinine sulfate in 0.05 mol/l H_2SO_4 as a reference. The QY was calculated with the following equation:

$$\Phi_x = \Phi_{st} \cdot (A_x/A_{st}) \cdot (F_{st}/F_x) \cdot (n/n_o)^2,$$

where Φ is QY, A is absorbance at the excitation wavelength, F is the integrated emission area across the band, and n is the refractive index of the solvent containing the samples (n) and the reference (n_o). The subscript "st" refers to the referenced fluorophore (quinine sulfate in 0.05 mol/l H_2SO_4) with known QY and "x" refers as the samples for the determination of QY. Absorbance of the sample and the reference was kept 0.100 ± 0.002 at the excitation wavelength of 350 nm.

3. Results and Discussion

For PAMAM modification, we applied two options for a simple one-step modification, which is based on the double cyclization reaction between the terminal fragments of PAMAM and CA (the schemes in Figs. 1 and 2). We considered different data while choosing an optimal temperature for the hydrothermal treatment of PAMAM mixture with CA. As previously reported, the formation of IPCA occurs at lower temperatures (100–150°C) than the formation of by-products of carbonization of CA and amine-containing agents. However, the yield of the product increases significantly with increasing synthesis temperature [17]. On the other hand, the possibility of PAMAM degradation with an increase of in temperature >120°C [15] demands limitation of the synthesis temperature. Thus, for the PAMAM modification, a temperature 120°C was used.

The absorption, excitation and normalized emission spectra of freshly prepared PAMAM aqua solutions are shown in Fig. 3, *a*. The absorption band of PAMAM is observed at 285 nm. The emission maximum of PAMAM is located in the region of 400 nm, while the effective excitation wavelength is 320 nm. The PAMAM fluorescence is a result of the dendrimers' structural peculiarities, which is caused an $n \to \pi^*$ transition from the amido groups throughout the dendritic structure [13]. To exclude an increase in the PAMAM fluorescence under the action of thermal treatment and increased pressure, an aqueous solution of PAMAM (without additives) was processed under the conditions according to the scheme in Fig. 2. The optical properties of PAMAM, including emission intensity, have not been change after both variants of heat treatments. This clearly indicates the integrity of the polyamidoimine structure under heating.

According to the previously described studies, [17–20], IPCA can be obtained by the double cyclization reaction of CA and EDA as a result of boiling under reflux at atmospheric pressure, but with a small reaction yield. However, IPCA with a higher reaction yield can be obtained using the hydrothermal treatment, but at the same time a larger amount of high molecular weight by-products of CA and/or 1,2-ethylenediamine polycondensation is formed [17, 19]. Therefore, this approach was not considered in our work. Absorption, excitation and normalized emission spectra of the resulting fluorophore (EDA-CA) are shown in Fig. 3, *b*. The absorption band at 350 nm corresponds to $\pi \to \pi^*$ or $n \to \pi^*$ transitions. The emission maximum of the obtained structures is located in the region of 450–455 nm, while the effective excitation wavelength coincides with the absorption band and is 350 nm.

The use of two approaches to the modification of PAMAM leads to products with the identical optical characteristics, presented in Fig. 3, *c*. As a result of the thermal treatment of PAMAM with CA, a long-wavelength shift of the absorption band to the region of 370 nm in comparison with PAMAM without modification is registered. The emission maximum of the obtained structures is located in the

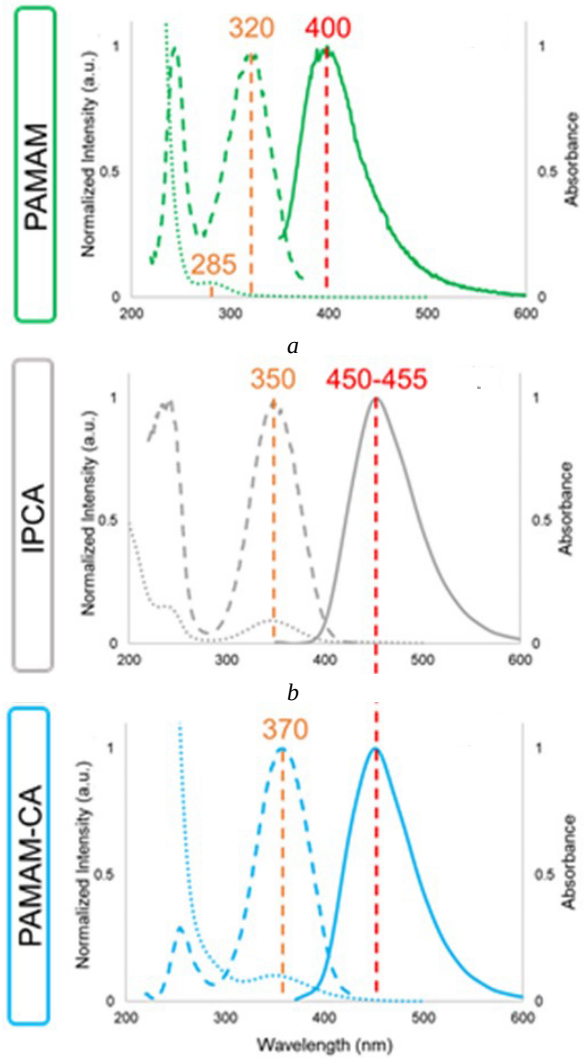

Fig. 3. Absorption (dot), excitation (dash) and normalized emission (solid) spectra of initial PAMAM (*a*), reaction product of EDA and CA (EDA-CA, IPCA) (*b*) and reaction product of PAMAM and CA (PAMAM–CA) (*c*) (color online)

region of 450–455 nm, while the effective excitation wavelength coincides with the absorption band and is 370 nm. The emission spectra of PAMAM–CA and EDA-CA (IPCA) are completely identical, that confirms the formation of the fluorophore at the terminal fragments of PAMAM. The change in the effective excitation band in comparison with the free fluorophore IPCA is most likely associated with the size of the fragment (the PAMAM core) associated with the imidozolidine fragment of the IPCA [17–20]. As a result of modification, the PAMAM fluorescence is significantly increased compared to PAMAM without modification.

Since there were no significant differences in the optical properties of the samples obtained by the two approaches, further analysis was carried out for the samples obtained by boiling under reflux at atmospheric pressure, because for this synthesis it is easier to control the reproducibility of conditions.

To evaluate the efficiency of emission, the relative QY was measured (Table). The quantum yield of the product obtained by boiling EDA and CA (IPCA) was $57 \pm 2\%$. After PAMAM modification with CA, the PAMAM QY increases to $28 \pm 3\%$. This is significantly lower than that for the free IPCA fluorophore. This may be due to the fact that PAMAM-bound IPCA molecules are in suboptimal conditions compared to free IPCA molecules in solution or the formation of the fluorophore does not occur at all PAMAM terminal groups.

Dynamic light scattering data were obtained to characterize the size and charge of the obtained samples (Table). The hydrodynamic diameter and ζ-potential of initial PAMAM was 3.4 ± 0.5 nm and 42 ± 5 mV, respectively, which is fully consistent with literature data [21–23]. The positive charge of PAMAM is due to the protonation of the surface primary amino groups. As a result of the PAMAM modification, there was no significant change in the hydrodynamic size, while the ζ-potential value was -24 ± 4 mV. A dramatical change in the ζ-potential of the PAMAM–CA compared to the initial PAMAM confirms the formation of a fluorophore. The presence of IPCA carboxyl group on the PAMAM surface forms a negative charge. However, for PAMAM with terminal carboxyl groups, the potential value is –40 mV [22], which is lower than the value obtained for the PAMAM–CA. This confirms our assumption that the fluorophore formation does not occur for all terminal groups.

Thus, the combined thermal treatment of PAMAM and CA, leads to the formation of a bright blue fluorophore (IPCA) at the terminal fragments

Table. Fluorescence quantum yield (QY), hydrodynamic diameter and ζ-potential of initial PAMAM, reaction product of PAMAM and CA (PAMAM–CA) and reaction product of EDA and CA (EDA-CA, IPCA)

Sample	QY, %	Hydrodynamic diameter, nm	ζ-potential, mV
PAMAM	< 1 [13]	3.4 ± 0.5	42 ± 5
PAMAM–CA	28 ± 3	3.5 ± 0.7	-24 ± 4
EDA-CA (IPCA)	57 ± 2	–	–

of PAMAM (emission maximum at 450–455 nm). Boiling under reflux PAMAM with CA makes it possible to increase the QY of fluorescence up to 28±3% from less than 1% of the initial PAMAM. The absence of changes in the hydrodynamic diameter of PAMAM–CA compared to PAMAM confirms the integrity of the polyamidoimine core.

4. Conclusions

As a result of the work, a simple one-step approach to the modification of PAMAM with a bright blue fluorophore (IPCA) has been proposed, which is implemented as a result of a double cyclization reaction between citric acid and terminal ethylenediamine fragments of PAMAM. Reaction of PAMAM and CA leads to the appearance of bright-blue fluorescence with a quantum yield of 28±3 %, which is significantly higher than the intrinsic fluorescence of PAMAM. A combination of bright fluorescence and a multifunctional terminal surface makes it possible to further use the obtained structures for biovisualization.

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