Assessing the Complex Formation between Crotamine—A Natural Cell-Penetrating Peptide—and DNA Using High Sensitive Fluorescence Exclusion Assay

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Abstract

Crotamine is a natural cationic polypeptide isolated from the venom of South American rattlesnake (Crotalus durissus terrificus) capable of forming complexes with DNA molecules by means of electrostatic interactions and translocating them across biological membranes. With such properties, crotamine can act as a transfection agent for several cell types but mainly in a stage of proliferation. However, to improve the biological use of this peptide, it is interesting to better characterize the formation and stability of crotamina-DNA complex. Thus, the present study aimed to analyze the stoichiometry, formation kinetics and protease stability of crotamina-EGFP complex using high sensitive fluorescent methodology, based on fluorescent exclusion assay. The best DNA:crotamine mass proportion for complex formation ranges between 1:10 to 1:40 as mass ratio. In 1:10 group, crotamine was able to complex about 75% of the available DNA as fast as in 20 seconds, and after 10 min of reaction crotamine reached its maximum complexation capacity, being able to complex 80% of total amount of DNA available. Under these conditions, we have determined that 1μg of crotamine can complex 80 ng of circular plasmid DNA (~4700 bp). Crotamine-DNA complex was resistant to proteinase K degradation even after twelve hours of enzymatic incubation. Additionally, the complex was sufficiently stable to trypsin digestion for a long incubation time: more than 73% of crotamine-DNA condensates maintained the integrity after 60 min, whereas 55% was intact after 180 min. Crotamine-DNA complexes were resistant to physiological temperatures, as well as stable for 15 days at 4°C and for two months when stored at -20°C. These results lead us to think that, in the best conditions of preparation proposed here, crotamine-DNA condensates can be used in an efficient and standardized manner as a potential agent of transfection in a transgenesis programme.

Keywords Crotamine; Cell-penetrating peptides; DNA condensates; Transgenesis

Abbreviations

CPPs: cell-penetrating peptides; EGFP, enhanced green fluorescent protein; HMW, high molecular weight; LMW, low molecular weight.

Introduction

Crotamine is one of the major components of South American rattlesnake Crotalus durissus terrificus that was originally described to cause spas tic paralysis when injected intra-peritoneally with high toxic concentrations [1]. Due to the amino acid composition, crotamine is a small basic polypeptide, encompassing 42 amino acid residues (4.8 kDa), from which eleven residues are basic (nine lysines, two arginines), conferring to the molecule a net positive surface charge and a high cationic character [2]. This natural cationic peptide is stabilized by six cysteine residues involved in three disulfide bonds [2,3] which allow for the formation of a typical scaffold (αβββββ) also adopted by the human beta-defensins.

Despite of the known neurotoxicity, Kerkis and collaborators [4] disclosed that crotamine, at low and non-toxic concentrations, possesses the intrinsic property of cell penetration and, consequently, a capacity of translocating eukaryotic cell membranes without causing any damage and, once inside the cell, localizing into the nucleus [5]. Despite of distinct mechanism of membrane translocation and cell uptake, most mediated by fluid phase endocytosis, CPPs have proved to be efficacious for carrying a number of biologically active and therapeutically relevant molecules inside the cells [9]. For this reason, CPPs have raised considerable interest as therapeutic and
biotechnological nanodevices and nanoshuttles [10]. Applications of so-called peptide transduction technology include drug and gene delivery, cell transfection, molecular diagnostic and adjuvant tumor therapy [11].

The fact that crotamine shows a promising applicability in efficient cell transfection has prompted us to assess the best in vitro conditions of crotamine-DNA complex formation, as well as check the stability of these condensates in solution. For this purpose, we based our experimental evaluation on a high sensitive fluorescence DNA exclusion technique, as reported here. Thus, the aim of this work was to characterize the kinetics of complex formation mediated by crotamine and to test the stability of DNA-peptide condensates in order to validate crotamine as peptide-based gene delivery system.

Materials and methods

Crotamine

Pure grade crotamine (>95%) was a gift from Prof. Eduardo B. de Oliveira (University of São Paulo, São Paulo, Brazil). Crotamine was purified by a combination of solvent precipitation and liquid chromatography, according to previously described by Kerkis et al. [4]. Lyophilized crotamine was usually stored at -20°C until use. Solubilized crotamine (1 mg/ml in deionized water, ~200 μM) was divided in aliquots of 100 μL and also kept at -20°C.

Preparation and monitoring of crotamine-DNA condensates

DNA-peptide condensation was formed using pure crotamine and the circular plasmid pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA), harboring a reporter gene encoding a green fluorescent protein. The initial experimental design was essentially as described in Nascimento, et al. [6]. The plasmid was propagated in Escherichia coli and then purified by PureLinkTM Quick Plasmid Miniprep Kit (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer’s instructions. The peptide and DNA mixture was incubated for 30 min in 0.15 M of NaCl at 26°C for condensate formation. The formation of DNA-crotamine complex was indirectly measured using “fluorescence exclusion assay”, based on the monitoring of the decreasing in the efficiency of fluorophore dye intercalation into DNA and in the light emission, which is assumed to be proportional to condensates formation. Thus, because DNA condensation by crotamine decreases double-stranded dye-intercalated DNA, measurements of the decreasing fluorescence signal allowed to indirectly accesses the proportion of condensed DNA. This approach was designed by our group as an alternative technique to others more expensive. The dye chemistry was that provide in the Quant-iTTM dsDNA kits (Broad Range, BR, and High Sensitivity, HS, versions) and the detection system was the Qubit® fluorometer both supplied by Invitrogen Co. The devised procedure was as follow: a 20 μL aliquot of crotamine and DNA was combined with 180 μL of Quant-iTTM working solution (buffer with fluorophore) and the non-condensed DNA concentration was accessed by fluorescence emission at 532 nm (excitation at 502 nm). Complementarily, samples of all DNA-peptide condensates were analyzed by electrophoresis on a 1% agarose gel prepared in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0), which were stained with ethidium bromide (0.5 μg/ml) and visualized in a UV light transilluminator. In all experiments (fluorescence exclusion and resolution with agarose gel electrophoresis), negative and positive controls were used. DNA without crotamina was used as negative control. DNA complex with lipofectamine (Invitrogen Co) was prepared according to manufacturer’s instructions and served as positive control.

Stoichiometry and kinetics of crotamine-DNA complex formation

At first, to determine the best DNA-peptide ratio, pEGFP-N1-crotamine complex was formed by combining a fixed concentration of crotamine (50 ng/μl) with increasing concentration of pEGFP-N1 (1.25, 2.5, 5.0, 10.0, 20.0 and 30.0 ng/μl), resulting in a DNA:peptide mass ratio of 1:40, 1:20, 1:10, 1:5, 1:2.5 and 1:1.7, respectively. The monitoring complex formation was performed with Quant-iTTM BR kit (at 0, 1, 10 and 30 min), as above described. Negative controls were prepared with DNA at the same concentrations described above but without crotamine and were used for fluorescence signal normalization. In a second set of experiment, the smaller concentration of DNA (1.25 ng/μl) was incubated with increasing amounts of crotamine (5.0, 25.0, 50.0, 250.0 and 500.0 ng/μl) to verify the kinetic of complex formation with high peptide mass ratios (1:4, 1:20, 1:40, 1:200, 1:400, DNA:peptide). Because the very low DNA amounts (less than nanograms), the Quant-iTTM HS kit was used for monitoring complex formation (at 0, 1, 10 and 30 min). Negative control was prepared with DNA without crotamine and was used for fluorescence signal normalization. In a third set of assay, the kinetics of complex formation was minutely monitored at a mass proportion of 1:10 (DNA:crotamine) by the fluorescence signal measurement at seven different times: 0, 1, 2, 3, 5, 10 and 30 min. Negative control was prepared with DNA without crotamine and were used for fluorescence signal normalization.

Proteolytic stability of crotamine-DNA condensates

DNA condensates (at mass proportion of 1:10, DNA:crotamine) were combined with 1.27 μg trypsin/μl (Invitrogen Co.) or 0.2 μg proteinase k/μl (Invitrogen Co.) and were incubated at 37°C for 12 h. Sample aliquots (20 μl) of reactions were taken at different time points (0, 15, 30, 60, 120, 180 and 720 min). Both fluorophore exclusion assay (with Quant-iTTM HS kit) and 1% agarose gel electrophoresis were used for complex formation analysis, as described above.

Results

In the first intent for stoichiometric assessment, realized with Quant-iTTM BR kit, we have observed that the best proportion for complex formation fits in the range of DNA:crotamine ratio comprised between 1:10 to 1:40 (w/w). In such ratios, crotamine was able to complex around 65% of the DNA (Figure 1). In the same assay, we observed that smaller quantities of crotamine (1:1.7 to 1:5; DNA:crotamine; w/w) were not efficiently and sufficiently able to complex the slight high amount of DNA, that resulted in lower percentage of condensed DNA (<40%; Figure 1). In addition, under the same condition of ionic strength and temperature, positive control (lipofectamine) was capable of making detectable complex, leaving only 41% of the all DNA available in solution (Figure 1). In Figure 2 is shown the results from the second set of stoichiometric assessment, which were also obtained by quantitative measurements with the Quant-iTTM HS kit and the specific fluorimeter. This set of experiments confirmed that the best DNA:crotamine proportion fits around 1:20 (w/w), since in this group crotamine was able to complex 75% of the DNA available in the mixture (1 μg of crotamine complex around 75 ng of DNA).
Figure 1: Kinetics of DNA-crotamine complex formation assessed by the fluorophore exclusion assay with Quant-iT™ Broad Range kit. Complexes were formed by combining 50 ng crotamine/µl with 1.25, 2.5, 5.0, 10.0, 20.0 or 30.0 ng pEGFP-N1/µl, resulting in 1:40, 1:20, 1:10, 1:5, 1:2.5 and 1:1.7 DNA:peptide mass ratios, respectively. Lipofectamine served as positive control. Negative controls were prepared with DNA at same concentrations described above but without crotamine.

Additionally, in the group of samples with higher amounts of crotamine (1:40 to 1:400; w/w), it was not observed an increase in the amount of condensed DNA, showing that additional amounts of crotamine (higher than 1:40) was not effective to increase the percentage complex formation (condensation around 80%). Intriguingly, other physico-chemical properties of crotamine molecule and DNA, such as repulsive charges and steric hindrance, might thus influence the efficacy of DNA-peptide complexation.

Figure 2: Kinetics of DNA-crotamine complex formation assessed by the fluorophore exclusion assay with Quant-iT™ High Sensitivity kit. Complexes were formed by combining 1.25 ng DNA/µl with 5.0, 25.0, 50.0, 250.0 and 500.0 ng crotamine/µl, resulting in 1:4, 1:20, 1:40, 1:200, 1:400, DNA:peptide mass ratios, respectively. Negative control was prepared with DNA without crotamine.

The evaluation of shorter time intervals in the formation of DNA-peptide complex allowed the observation that in the first time point (20 seconds of incubation) less than 25% of the DNA was left uncondensed. In a time as short as 10 min of reaction, crotamine reached its maximum capacity of DNA condensation, being able to complex 80% of the amount of DNA in solution. Under these conditions, in which DNA:crotamine ratio equals 1:10, one can infer that 1µg (~0.2 nmoles) of crotamine can effectively complex 80 ng of DNA (Figure 3).

The DNA-peptide complex formation, for all aforementioned ratios, was confirmed by DNA mobility shift in agarose gel electrophoresis (Figure 4). All negative control groups, with different amounts of circular “naked” plasmid (1.25, 2.50, 5.0, 10.0, 20.0 and 30.0 ng/µl), separated well in agarose/TAE gel, showing the typical pattern of multiple coiled bands, but with expected molecular weight around 4,700 bp and ethidium bromide fluorescent intensity proportional to DNA concentration (Figure 4). As expected, in virtue of complexation of DNA by crotamine, an appreciable band shift was observed in the agarose gel (Figure 4; PC groups), with a complete halt in the nucleic acid migration and, consequently, the electrophoretic mobility. Such phenomenon occurs due to a neutralization of negative charge in DNA molecule, caused by the association of highly cationic crotamine, once the complex is formed. Moreover, in the experimental groups composed of crotamine-DNA in which plasmid is mixed in excess (20 and 30 ng/µl) with fixed amount of crotamine, it can be observed that remaining non-condensed DNA is detected in agarose gel (Figure 4; gray squares) and in the fluorescence exclusion assay (Figure 1; 1:1.5, 1:2.5).

The protease stability of crotamine-DNA complex was examined using two kinds of proteases: trypsin and proteinase K. The
fluorescence exclusion revealed that crotamine-DNA complex was resistant to proteinase K degradation even after twelve hours (720 min) of incubation (Figure 5A). Interestingly, 73% of crotamine-DNA complex was stable after 60 min of incubation with trypsin, and 55% of DNA condensates have their integrity preserved even after 180 min of proteolytic exposition. However, only 17% of complex was quite stable in a period of twelve hours of digestion (Figure 5B). Analytical cross checking by means of agarose electrophoresis (Figure 6) confirmed the results obtained with fluorometric assay. In fact, only after 12 hours of incubation with trypsin, the proteolytic action was observed (Figure 6, panel C, lane 4).

Discussion

Nucleic acids, like plasmid DNA (pDNA), can be vectorized with natural and/or synthetic, cationic peptides, lipids or polymers [12,13]. The common characteristic between all these classes of molecules is their capability of charge-interaction with DNA and its condensation into nanoparticles, which are suited for intracellular delivery [14].

CPPs compose a prominent group of nonviral peptide-based vehicles, which have been shown to facilitate the delivery of DNA and other macromolecules. Several research groups worldwide have dedicated efforts to describe the vectorization of pDNA with CPPs, although the overall efficiency has been often low [15,16]. This fact is a critical point for the application of CPPs in transduction technology for transfection and transgenesis. One of the factors that influence the efficiency of transfection is the stoichiometry relation between these delivery agents and the DNA of interest [17].

Therefore, in the present study, we assess in solution the stoichiometry and kinetic of complex formation between crotamine and plasmid DNA. With this aim, we devised a simple but reliable and sensitive technique based on fluorescence exclusion of DNA intercalating dye. The principle underlying this technique involves the DNA condensation ability of crotamine and the selective intercalating fluorophore contained in the Quant-iT™ dsDNA (Broad Range or High Sensitivity) kit. In this kit, the fluorescent dye is sensitive enough to detect minute amount and concentration (10 pg/µl to 100 ng/µl) of double stranded DNA. With the correct stoichiometry, crotamine promotes nucleic acid condensation and, in this manner, fluorescence titration mediated by the exclusion of intercalating fluorophore (from DNA molecule) can be achieved and would correlate with complex formation. The fluorescence readings are then conducted in real time using fluorimeter equipment. This simple and reliable assay is an alternative to methodologies that involve “fluorescence quenching” and “light scattering” for our purpose of investigating the interaction of cationic peptide (crotamine) and nucleic acid (plasmid). A confirmation that our approach was working well, band (or mobility) shift technique was used in combination with fluorescence exclusion readouts.

According to our findings using these alternative and combined techniques, crotamine initiates DNA complexation very fast (~20 sec) and in 10 min the complex formation is maximal. The optimal ratio of peptide (crotamine) and DNA (plasmid) masses lies between 1:10 and 1:40, indicating that 1μg of crotamine is able to make complex with up to 80 ng of plasmid DNA (~4700 bp). It is interesting to note that DNA tested in the present study is a circular plasmid harboring a reporter gene (EGFP), which is currently used as control in experiments of transfection. In fact, in previous published works, crotamine-mediated transfection was successful achieved in a number of eukaryotic cells [4,6-8].

Another significant finding is the fact of complexes formed by crotamine and DNA is resistant to proteases (trypsin and proteinase K) for relatively long period of incubation (up to 12 h). It is known that peptides stabilized by multiple disulfide bounds are resilient to protease attack, and more surprising as seen here is that the “core of crotamine” does not promptly expose condensed DNA upon proteolytic digestion.

Seen that crotamine is taken up by the cell through an endocytic pathway, being subsequently accumulated in lysosomal vesicles, it is argued that crotamine must resist to proteolysis, at least partially, and protect the cargo DNA molecules. In fact, to be effective as an efficient delivery vehicle in biotechnological procedures (e.g., transgenesis), crotamina-DNA complex should resist to attack and digestion of several lysosomal proteases. Thus, CPPs and other cationic molecules
that possess the ability to protect the cargo DNA molecule from extra and/or intracellular milieu have obvious extra advantage for application as efficient gene delivery system [18,19].

Furthermore, the endosome compartment has been considered one of the major cellular barriers that limit the gene transfer efficiency by non-viral gene delivery systems [20]. To increase the endosomal escape of DNA, several research groups have studied the incorporation of fusogenic peptides into experimental delivery systems [21]. While this approach often leads to significant increases in gene transfer efficiency in vitro, ionic interactions are relatively weak, leading to premature dissociation and degradation in vivo of the carrier DNA [22]. Another interesting point to take into account is that endocytosed macromolecules must travel through a reducing environment to reach the nucleus and delivery a gene. McKenzie and collaborators [19] suggested that disulfide bonds could transiently stabilize peptide-DNA condensates and once the peptide is reduced, the intracellular release of DNA is initiated. Moreover, it has been established that condensate stability increases coincidentally with the number of cysteine residues, whereas the size of the condensate decreases. Seen that crotamine have six cysteine residues involved in tree disulfide bonds [10], we can infer that this small cationic peptide can form strong condensate with nucleic acid and improve the transfection rate. It is estimated that 13 molecules of crotamine interact to and complex one molecule of plasmid DNA (~4700 bp). These additional properties qualify crotamine still more as an attractive vehicle for transduction technology and transgenesis.

In conclusion, in this study we have devised a practical method to assess the kinetic formation of crotamine-DNA condensates and other peptide-nucleic acid complexes, showing that at non toxic concentrations of this peptide can rapidly and efficiently form complexes with nucleic acid molecules (plasmids). By means of fluorescence exclusion assay we have determined that the optimal mass ratio for crotamine-DNA complex formation. Additionally, we have certified that crotamine-DNA condensates are stable to physiological temperatures and proteases digestion. Altogether, these findings allow us to further investigate and apply crotamine as a feasible and reliable vehicle to improve the efficiency of some biotechnological processes, for example, in the animal transgenesis.

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