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Fluorescence Correlation Spectroscopy to find the critical balance between extracellular association and intracellular dissociation of mRNA complexes

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ABSTRACT

Fluorescence Correlation Spectroscopy (FCS) is a promising tool to study interactions on a single molecule level. The diffusion of fluorescent molecules in and out of the excitation volume of a confocal microscope leads to the fluorescence fluctuations that give information on the average number of fluorescent molecules present in the excitation volume and their diffusion coefficients. In this context, we complexed mRNA into lipoplexes and polyplexes and explored the association/dissociation degree of complexes by using gel electrophoresis and FCS. FCS enabled us to measure the association and dissociation degree of mRNA-based complexes both in buffer and protein-rich biological fluids such as human serum and ascitic fluid, which is a clear advantage over gel electrophoresis that was only applicable in protein-free buffer solutions. Furthermore, following the complex stability in buffer and biological fluids by FCS assisted to understand how complex characteristics, such as charge ratio and strength of mRNA binding, correlated to the transfection efficiency. We found that linear polyethyleneimine prevented efficient translation of mRNA, most likely due to a too strong mRNA binding, whereas the lipid based carrier Lipofectamine® messengerMAX did succeed in efficient release and subsequent translation of mRNA in the cytoplasm of the cells. Overall, FCS is a reliable tool for the in depth characterization of mRNA complexes and can help us to find the critical balance keeping mRNA bound in complexes in the extracellular environment and efficient intracellular mRNA release leading to protein production.

Statement of Significance

The delivery of messenger RNA (mRNA) to cells is promising to treat a variety of diseases. Therefore, the mRNA is typically packed in small lipid particles or polymer particles that help the mRNA to reach the cytoplasm of the cells. These particles should bind and carry the mRNA in the extracellular environment (e.g. blood, peritoneal fluid, ...), but should release the mRNA again in the intracellular environment. In this paper, we evaluated a method (Fluorescence Correlation Spectroscopy) that allows for the in depth characterization of mRNA complexes and can help us to find the critical balance keeping mRNA bound in complexes in the extracellular environment and efficient intracellular mRNA release leading to protein production.

1. Introduction

Nucleic acids have been explored widely in basic research and biomedical applications in the past decades. There is a growing interest in the use of mRNA as a potential therapeutic in various medical indications, ranging from hereditary or acquired metabolic diseases to regenerative diseases, therapeutic cancer vaccination and protein-replacement [1]. Conceptually, mRNA exerts its function in the cytoplasmic compartment and does not depend on nuclear envelope breakdown. Also, unlike plasmid DNA (pDNA), there is no risk of insertional mutagenesis for mRNA, which provides a substantial safety advantage for clinical practice [2–4]. In addition, mRNA production is relatively simple and low-cost as

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there is no need to select and incorporate a specific promotor into the transfection construct [5].

To advance protein-replacement therapies [1,6–10], efficient mRNA delivery to the target cells is key. *In vitro* transfection strategies have benefited from the development of formulations to protect mRNA from degradation mediated by RNase and to facilitate cellular uptake [11]. In addition to the cationic lipids [12–16], other investigated chemical delivery systems include cationic polymers, such as polyamidoamine dendrimers and polyethylenimine (PEI).

Among the various types of non-viral vectors, cationic lipids are especially attractive as they can be prepared with relative ease and have been extensively characterized [17,18]. More and more commercial non-viral vectors consisting of cationic lipids are available for *in vitro* mRNA delivery, such as Lipofectamine® 2000 and Lipofectamine® messengerMAX [19–21]. In contrast, cationic polymers are hardly investigated for mRNA delivery, although they have been widely exploited with great success for other nucleic acids, such as pDNA, oligonucleotides and siRNA [22]. One of the most widely employed cationic polymers is PEI, due to its advantages such as a strong DNA condensation capacity and buffer capacity, which provides PEI with some endosomal escape ability (proton sponge effect). PEI exists as a linear or branched polymer and can be found with different molecular weights. Among them, linear PEI of 22 kDa and branched PEI of 25 kDa are the most effective transfection reagents used *in vitro* and *in vivo* with pDNA [23,24]. However, high molecular weight PEI also shows relatively considerable toxicity, whereas PEI with low molecular weight has low toxicity but less transfection efficiency. It has been reported that PEI polymers are able to efficiently complex and thus protect unmodified RNA molecules including ribozymes and siRNAs [25]. Only few studies addressed PEI for mRNA delivery, where it was typically less effective [22], potentially due to differences in mRNA-polycation binding compared to pDNA and siRNA [26–29].

Both cationic polymers and cationic lipids face barriers to gene delivery [30]. The mRNA delivery systems should have high transfection efficiency, low cytotoxicity and no immune response [31]. Also, it should achieve a sufficiently high level of the encoded protein and reach a high number of cells. In most transfection studies, complexes are administered to cells in a reduced serum condition such as Opti-MEM when evaluating their stability, uptake, intracellular trafficking and transfection. It is well-established, however, that once in contact with protein-rich biological fluids, most nanoparticles are spontaneously covered by a layer of biomolecules with the formation of a so-called "protein corona" [32–34]. This corona might seriously affect the performance of complexes, both on the level of extracellular stability, cell targeting, cellular uptake and intracellular trafficking [35,36]. Therefore, performing *in vitro* optimization of nano-sized formulations in undiluted biological fluids before assessing the functionality *in vivo* is always advised [37]. Clearly, a good delivery system should maintain a critical balance between complexing the mRNA in the extracellular environment, but releasing it inside the cytoplasm of the cells. The association and dissociation from mRNA to a certain carrier is mostly investigated by gel electrophoresis. This analytical tool, however, is not applicable to explore the stability and integrity of mRNA-carrier complexes in biological fluids, as proteins and nucleic acids in the biological fluid interfere with the read-out of the gels [26–29,38–40].

Our research group has reported before that FCS is a valuable tool to explore the delivery of short antisense oligonucleotides and siRNA (21 nucleotides) with lipid-based and polymer-based carriers: both the protection of the nucleic acids against enzymatic degradation and the association and dissociation of the nucleic acids from the carriers can be followed in buffer, in biological fluids such as human serum and intraperitoneal fluids and inside living cells [41–43]. The applicability of FCS to explore complex characteristics between carriers and much longer nucleic acids such as mRNA (1000 nucleotides), however, has not been reported before. In the present study, we aimed to evaluate whether FCS can be used to explore the stability of mRNA-complexes in buffer and in biological fluids. Lipofectamine® messengerMAX and linear PEI were chosen as representative lipid- and polymer-based carriers to complex mRNA into lipoplexes and polyplexes, respectively. We found that FCS is able to measure the degree of association and dissociation of mRNA-based complexes in buffer, full human serum and human ascitic fluid in a few minutes, while gel electrophoresis resulted in reliable measurements only in buffer. Furthermore, we evaluated the transfection efficiency of the mRNA-complexes in low-protein and high-protein conditions and attempted to correlate the results with differences in mRNA binding and release, as determined by FCS. The lipid-based carrier used in this study was more efficient when compared to linear PEI that failed to release the complexed mRNA in the cytoplasm of the cells. Therefore, a critical balance should be maintained between complexing the mRNA in the extracellular environment, without compromising the ability of the carrier to release its cargo into the cytoplasm of the cells.

2. Materials & methods

2.1. Consumables

Lipofectamine® MessengerMAX and linear jetPEI (average Mw 22 kDa) were purchased from Invitrogen (Merelbeke, Belgium) and Polyplus-transfection® SA (Strasbourg, France), respectively. mRNA encoding GFP labeled by Cyanine 5 (ARCA capped, 25% Cyanine 5-U, 75% Pseudo-U, 100% 5-Me-C) was purchased from Trilink (California, USA). HEPES, sucrose, sodium dodecyl sulfate and dextran sulfate with Mw's of 50 kDa were purchased from Sigma-Aldrich (Belgium). Mccoy's 5A modified Medium, Opti-MEM, 0.25% Trypsin-EDTA (1×), penicillin–streptomycin (5000 U/ml) and DPBS [−] (no calcium, no magnesium) were purchased from Invitrogen (Merelbeke, Belgium).

2.2. Collection of biological fluids

Human serum was obtained from healthy volunteers. Blood was collected into Venosafe™ 6 ml tubes containing gel and clotting activator (Terumo Europe™, Leuven, Belgium) at Ghent University hospital. Then the tubes were centrifuged for 10 min with a speed of 4000×*g* at 20 °C. The supernatant (serum) was aliquoted (50 μl) in sterile polypropylene tubes and stored at −20 °C and thawed at 4 °C overnight prior to use. Human ascitic fluid was obtained from patients diagnosed with peritoneal carcinomatosis at the department of medical oncology, Ghent University hospital, with approval of the ethics committee of the Ghent University hospital (ECD no. 2013/589).

2.3. Association degree (%) of complexes in HEPES buffer measured by gel electrophoresis and FCS

To study the complexation of mRNA and linear PEI (linPEI) by agarose gel electrophoresis and FCS, 5 μl PEI solution (the concentration of PEI depending on the desired N/P ratio of 0, 0.25, 0.5, 1, 2, 5, 10, 15, 20, 30) was added to 5 μl mRNA (80 μg/ml), vortexed for 15 s and incubated at room temperature for 30 min prior to use. messengerMAX/mRNA complexes were prepared as following: messengerMAX solution (the volume depending on the desired v/w ratio (volume (μl)/weight (μg)), namely 0, 0.25, 0.5, 1, 2, 3, 5, 10) was mixed with HEPES buffer (pH7.4, 20 mM) to a total volume of 5 μl and incubated at room temperature for 10 min prior to use.

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