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Stability of Free and Liposomal Encapsulated RNA on a Mucoadhesive PVA Polymer for Esophageal RNA Drug Targeting Using the EsoCap System

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The development of RNA and oligonucleotide-based therapeutics is a longstanding goal and is currently gaining significant attention. Several RNA-based drugs are approved for clinical use. Others are under investigation or in preclinical trials. This study have initiated the development of RNA drugs for localized use in the esophagus, utilizing the EsoCap System. This system's core component is a mucoadhesive film that carries the RNA drug and is precisely applied to the esophagus. Research into the stability and properties of naked and liposomal-encapsulated RNA on mucoadhesive polymer film reveals that RNAs remain stable in various conditions without degradation, RNA leakage, or liposome fusion observed. The liposome size also remains constant after application on the film, drying, and rehydration. These findings pave the way for RNA drug development for esophageal diseases and their administration via the EsoCap system.

1. Introduction

The development of innovative advanced therapeutic options to treat, palliate and prevent diseases in our society is dependent on novel and highly effective active substances. For drug therapy in patients, there are various ways to establish innovations which also often lead to, for example, faster recovery or improved compliance. On the pharmacological side, the development of novel active substances improves the therapy of diseases.[1] From a technological perspective, advanced application forms ensure an improved use of medicines, an increase in compliance and often a decrease in duration of treatment.[2] However, even the best and most innovative active substances do not achieve superiority as long as there is no biorelevant possibility of drug delivery and application.[3]

A particularly significant and promising platform technology is, for example, lipid nanoencapsulation in combination with RNA technology, which is already being used worldwide for immunization against the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS Cov-2) virus when applied intramuscularly. An also promising technology is the EsoCap concept for local and long-term therapy of esophagus diseases, which has been investigated in a clinical phase II trial.[3,4] Innovative active ingredients necessitate innovative dosage forms and advance their further development, as fresh forms of dosing entail a potential for innovative active components.[3,5] In the field of RNA technology, chemically unmodified nucleic acids have a very short half-life due to their rapid degradation by nucleases and are also eliminated by the body's own immune system, which is able to recognize foreign ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).[6] Furthermore, solubilized nucleic acids injected into the body were largely ineffective because the nucleic acids must be delivered into the cytoplasm (mRNA, siRNA, antisense oligonucleotides) or the nucleus (DNA, CRISPR-RNA) of the cell, to bind their target or, in the case of mRNA, to be translated to protein.[6b] These challenges generated several innovations, such as the modification of nucleic acids to provide resistance to nuclease degradation, reduced immunogenicity and increased interaction with the target cell.[7] In 2018, the first lipid nanoparticle-based siRNA therapeutic, *Onpattro*, was approved for the treatment of polyneuropathy.[8] In addition to the further development of nucleic acids, it was also possible to draw on decades of development work on lipid-based nanoparticles for use in humans and on PEGylation.[9] The development, emergency use authorization and large-scale use in the USA of the nucleoside-modified

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mRNA vaccines from BioNTech/Pfizer (*Comirnaty*/BNT162b2) and Moderna (*Spikevax*/mRNA-1273), in which the RNA encodes the SARS Cov-2-specific spike protein, can be seen as a breakthrough innovation for this technology.[10] As with *Onpattro*, lipid nanoparticles with ionisable cationic lipids serve as the delivery platform for BioNTech and Moderna's vaccines. These lipids have been optimized over decades and make a significant contribution to successful immunization.[10] The development of mRNA vaccines is a particularly topical example of the connection between the success of a therapy, the development of active substances and the use of suitable dosage forms.[3,5]

In the field of esophageal therapy, the particularly short transit time through the ≈25 cm long muscular tube posed a major challenge for local treatment.[11] In addition, the esophagus has a kind of self-cleaning effect through peristalsis.[12] In studies on the residence time of syrups or other highly viscous preparations, Hefner et al. were able to measure a residence time of only a few minutes on the mucosa of the esophagus by means of scintigraphy.[13] However, the efficacy of local esophageal therapy is directly related to the residence time of the dosage form at the site of application, as shown by Dellon et al. in a study of patients with eosinophilic esophagitis using preparations of different viscosities.[14] The EsoCap concept was developed to address these challenges.[4a] It consists of a mucoadhesive, thin polymer film that is rolled up and inserted into a commercially available but slotted hard gelatine capsule.[4a] The free end of the film that protrudes from the capsule is connected to a thread called a retainer, which is responsible for the exact delivery of the system as a trigger mechanism.[4b] There is also a placebo weight inside the capsule to facilitate the swallowing process.[4b] The capsule with the film is placed in a special applicator to which the free end of the retainer is attached. The applicator, which resembles a beak cup, is filled with water so that when the device is taken, the capsule falls out of the applicator into the patient's throat, where it is swallowed together with the water.[4b] Once the retainer is expanded to the maximum, the film is pulled out of the capsule during transport through the esophagus and placed there. Afterwards, the film begins to dissolve and forms a mucoadhesive gel, from which the drug can be easily released.[4b] The process resembles an eroding matrix and is not a diffusion-controlled release. Furthermore, mechanical factors such as esophageal peristalsis also contribute to the drug penetrating from the gel into the mucous membrane. The EsoCap system represents a completely new technology for the application of films in the esophagus, although films for local application to buccal or vaginal mucous membranes loaded with small molecules are already available.[4a,d] There are a number of diseases of the esophagus that could potentially be treated depending on the loading of the film. In a Phase II study, the number of eosinophil peaks in inflammatory diseases, particularly eosinophilic esophagitis, was significantly reduced.[3,4c] Other diseases of interest for the device, include Barrett's esophagus, cancer, spasticity and possibly gastroesophageal reflux disease (GERD).

The combination of the two existing innovative platform technologies in form of an RNA-loaded mucoadhesive film that can be placed locally in the gastrointestinal tract could have a major impact on the therapy of patients with diseases of the esophagus in the future. The aim of the following study was therefore to apply and stabilize free and liposomal encapsulated RNA onto a mucoadhesive polymer film that can potentially be placed on the mucosa of the esophagus, e.g. by using the EsoCap drug delivery concept. Initial experiments were carried out with free RNA to investigate how drying of the RNA on the EsoCap film affects RNA stability. RNA as a polyanion presents a challenge for cellular transfer due to its negative charges and thus, potentially poor membrane permeability.[15] Therefore, for improved future transfection and cellular delivery, RNA encapsulated in liposomes was included in the study. This should not only facilitate more efficient transfer, but can also provide protection against ubiquitous nucleases present in the human body.

2. Results

2.1. Stability of Free RNA on the EsoCap Film

Using the EsoCap system for RNA application requires prior investigation of the stability of RNA on the EsoCap film. An arbitrarily chosen RNA Sequence A was used for these initial investigations. In a parallel experiment, the same RNA labelled at its 3′-terminus with the dye ATTO633 (Sequence B), was studied.

Sequence A	5′-AUUUCGAGUUGGCUGUUGCUU-3′
Sequence B	5′-AUUUCGAGUUGGCUGUUGCUU-ATTO633-3′

For the stability studies, both RNA samples were applied to the film using different methods. Initially, the RNA solution was applied directly onto the moist film immediately after distributing the viscous polyvinyl alcohol (PVA) solution (**Figure 1**, Method 1). Alternatively, the freshly prepared film was dried overnight, and the RNA solution was pipetted onto the dry film (Method 2). The purpose of this test was to determine whether applying the RNA onto the wet film would result in a smoother film, which would be more beneficial for later stages in drug production. To test the stability over an extended period of time, sample B was subjected onto the dry film and stored on the film at 4 °C for 10 days before analysis (Method 3).

The film pieces were treated with the RNA following Method 1 or 2, dried, then dissolved in water. The resulting solutions were analyzed on a polyacryl amide (PAA) gel. As seen in Figure 1A, RNA bands in all lanes are identical, indicative of RNA stability under all tested conditions. It is important to note that application of the RNA can occur on the moist (lane A1) or dry film (lane A2) without detectable degradation. The application on the moist film produces a smoother looking film, what may be an advantage for further drug formulation. When the labeled RNA B was used, detection is much more sensitive, and also smallest amounts of degradation products would become visible. Again, the RNA was subjected on either the moist or the dry film, the samples were dried followed by rehydration and analysis on a PAA gel with fluorescence detection (Figure 1B). No additional bands were observed in the gel (lane B1 and B2), showing that no detectable degradation had occurred.

To determine the stability of the RNA on the EsoCap film over longer periods of time, RNA B was subjected onto the dry film, dried and stored for 10 days at 4 °C prior to analysis. As seen in Figure 1C, this experiment confirmed the superior stability of

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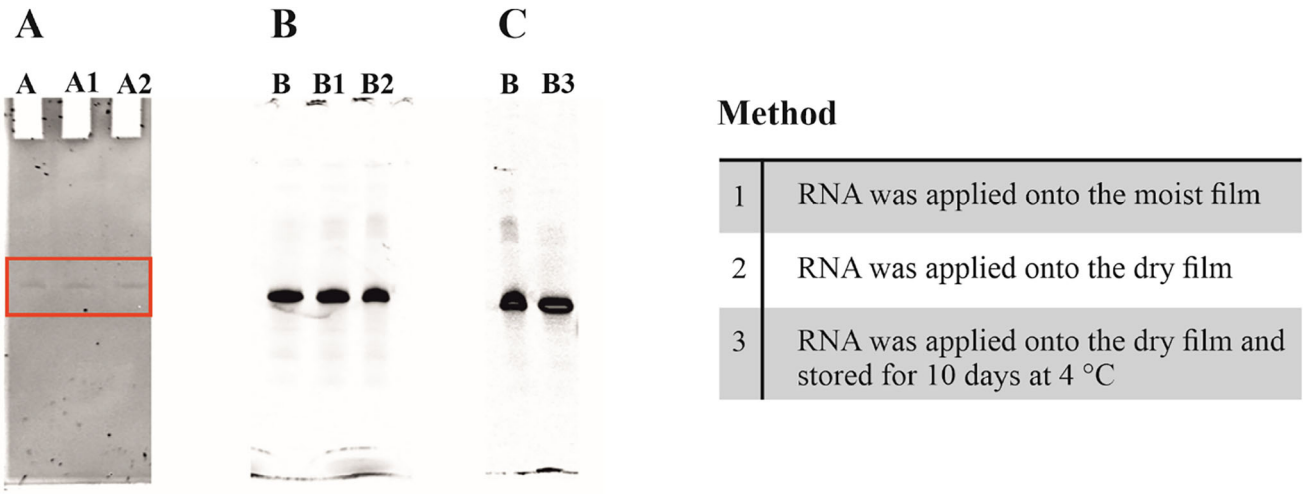

Figure 1. 20% PAA-Gels A) Lane A – RNA A as reference, lane A1 – rehydrated film treated with RNA A following Method 1, lane A2 – rehydrated film treated with RNA A following Method 2; stained with SYBR^{TM} gold; B) Lane B – RNA B as reference, lane B1 – rehydrated film treated with RNA B following Method 1, lane B2 – rehydrated film treated with RNA B following Method 2; visualized by fluorescence at 675 nm; C) Lane B – RNA B as reference, lane B3 – rehydrated film treated with RNA B following Method 3; visualized by fluorescence at 675 nm.

the RNA on the EsoCap film. No additional bands indicative of degradation were detected.

2.2. Application of RNA Encapsulated in Liposomes on the EsoCap-System

Liposomes were prepared following the thin layer hydration method. We used dioleolylphosphatidylcholine (DOPC) dissolved in trichloromethane. After evaporation of the solvent and drying, the obtained lipid film was rehydrated with Tris-HCl buffer (pH 8) containing 250 mM sucrose. Sucrose was added due to the drying step required after applying the liposomes to the EsoCap film. The disaccharide is commonly used in applications that involve drying of liposomes in order to ensure liposome stability and prevent release of encapsulated material.[16] This is due to the sugar induced vitrification of the liposomes, resulting in a barrier between individual lipid vesicles and limited mobility. As a result, liposome fusion is prevented. At the same time, water-lipid interaction is minimized, because of the sugar molecules replacing water molecules at the liposomal surface. Furthermore, the lipid bilayer transition temperature is reduced, making release of the encapsulated material less likely.[17]

The liposomes produced under those conditions were frozen in liquid nitrogen and subsequently thawed at 54 °C in eight repetitions. Finally, they were homogenized by extrusion (for details see Section Analysis of Free RNA upon Drying on the Film).

First, we looked at the features of "empty" liposomes (without RNA) on the EsoCap film. After subjection of liposomes (10 µL) (solved in phosphate buffered saline (PBS) containing 250 mM sucrose) onto the film, the film was dried, immediately rehydrated in water and analyzed using dynamic light scattering (DLS) (**Figure 2**). While the free RNA did not show any significant differences between drying on a still moist or dry polymer (Figure 1), in the case of liposomes, a noticeable difference was observed. Drying the liposomes on a still moist polymer (Figure 2A) resulted in a stronger dispersion of the particle diameter determined from three independent experiments, compared to application and drying on a dry polymer (Figure 2B). For the latter, the liposome size was found being the same in all three measurements, although a slight broadening of the liposome peak in comparison to the measurement before drying (grey curve in Figure 2B) occurred. In both measurements (Figure 2A,B), a new peak corresponding to particles having a diameter of ≈10 nm was observed, which was identified as resulting from the polymer itself. Note, that the same polymer film used in the experiments with the free RNA was employed here. The polydispersity indices for all measurements were greater than 0.5, indicating a non-homogeneous solution (this is expected, as two particle fractions, the polymer PVA and the liposomes, are present). Therefore, we used the correlation curve for evaluation of the measured data, and the smooth autocorrelation functions (on the right side of Figure 2A,B) suggest that the measurements were of high quality and reliable.

Next, we investigated the stability of RNA encapsulated in liposomes on the EsoCap film. Therefore, RNA A was encapsulated using a protocol for active encapsulation and a lipid end concentration of 1 mg mL^{-1}.[18] For separation of free RNA from liposomal encapsulated RNA, we tested a number of techniques including size exclusion chromatography on Sephadex G-25 and G-50, dialysis through an 8 kDa membrane for 48 h, and sucrose density gradient ultracentrifugation. These methods did not give satisfying results, as sufficient separation of free RNA from liposomal encapsulated RNA was not achieved. Separation by magnetic beads coated with a strong anion exchanger delivered much better results and thus was finally the method of choice. The free RNA was bound to the quaternary aminoethyl groups on the magnetic beads surface and thus, with the help of a magnetic separator, was separated from the liposomal encapsulated RNA. As a result, liposomes with an average size of 191.7 nm and a polydispersity index (PDI) of 0.21 (**Figure 3A**) were generated, slightly larger than empty liposomes generated before encapsulation (140, 7 nm, Figure 2A). For calculation of the encapsulation efficiency (EE), we used the RiboGreen assay (Figure 3B). Based

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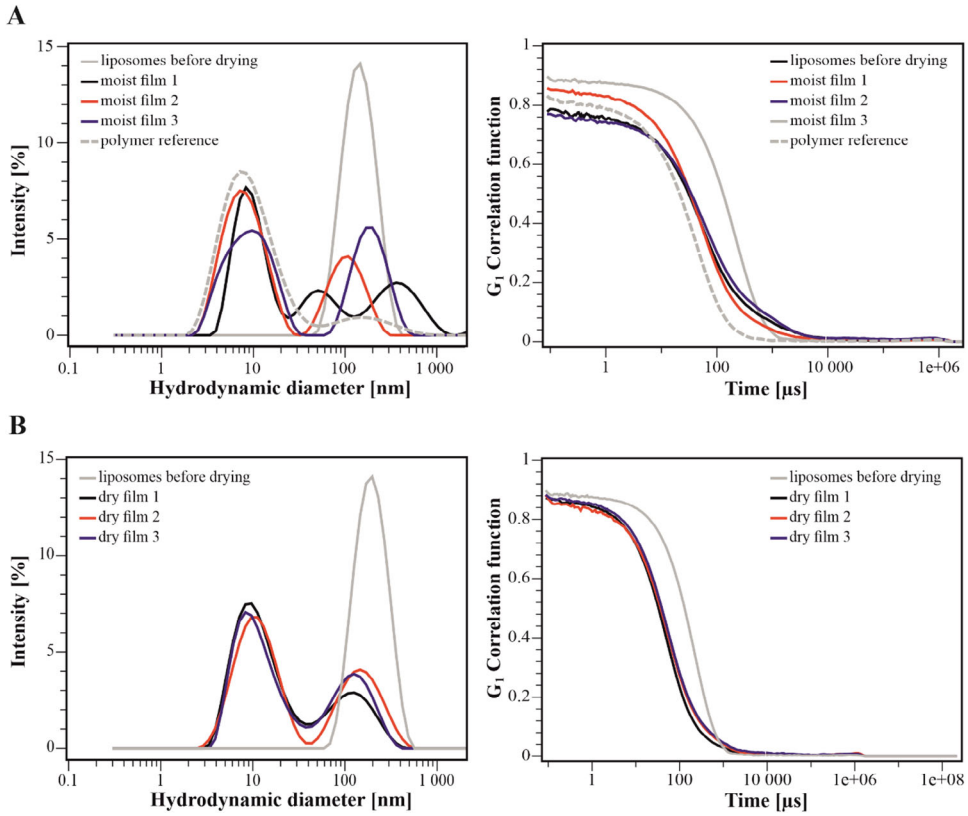

Figure 2. DLS data showing the hydrodynamic diameter (left) and the corresponding autocorrelation function (right) of the liposomes after subjecting onto the moist A) or dry B) polymer film, drying and rehydration, (three independent experiments were carried out, resulting in the black, red, and blue curves in panel A and B) in comparison to liposomes before drying (grey curve in A and B).

on the fluorescence of the dye upon binding to free RNA, the amount of RNA was determined in the absence and presence of tritonX-100 to destroy the liposomes, and the EE was calculated following Equation 1 (see Section Determination of EE). An EE of 10.7% was obtained, corresponding to 0.8 nmol of encapsulated RNA.

In a next series of experiments, the liposomal encapsulated RNA was subjected onto the film, and liposome size and RNA retention were analyzed. Here we exclusively subjected the liposomes onto dry films, because of the better size stability of liposomes on dry films as compared to the size variation observed when applied on the moist film (see above). As a control, liposomes with their RNA cargo were dried in the absence of the polymer film in a test tube cap, then rehydrated and measured by DLS (black line, **Figure** 4A). Liposomes before drying are represented by the grey curve (Figure 4A). Then, three experimental conditions were tested: i) Liposomes in sucrose containing buffer were subjected onto dry film (containing no sucrose), dried, rehydrated with water and measured (red curve in Figure 4A). ii) Liposomes in sucrose containing buffer were subjected onto dry film containing the same concentration of sucrose as the liposome sample, and dried (dark blue in Figure 4A). iii) Liposome sample as in *(ii)*, for enhanced protection was additionally diluted with the same volume of sucrose containing buffer before subjection onto the film and drying, (bright blue curve in Figure 4A). In all samples, with the exception of the control, in which the liposomes with their RNA load were dried in a test tube lid in the absence of the polymer film (black curve), a new signal was detected at ≈10 nm after drying and rehydration.

Furthermore, the DLS curve corresponding to the liposomes applied to the film without sucrose (red) shows an additional shoulder at ≈43 nm. The main peak of all samples was found in the area from 99 to 262 nm, showing quite some variation in liposome size, dependent on the specific experimental conditions. Prior to drying, the liposomes had a mean hydrodynamic diameter of 192 nm, which upon drying (in the control, black) slightly increased to 262 nm. Liposomes applied onto the film without sucrose, upon drying and rehydration maintained their mean size at 187 nm. In the samples however, that were applied onto the film containing sucrose, the mean size of the liposomes was reduced after the drying/rehydration process to 125 nm (dark blue) and 99 nm (sucrose in the film and sample diluted 1:1 with protection buffer (250 mM sucrose in PBS) before drying), (bright blue). As already seen in the experiments with empty liposomes (Figure 2), PDIs of greater than 0.5 were obtained, indicating inhomogeneous samples when liposomes and the EsoCap polymer were present, while in the control experiment, where the sample was dried without the EsoCap film, the PDI was 0.13. However, the autocorrelation functions were smooth in all cases, indicating reliable measurements of high quality.

For the analysis of RNA retention during the drying and rehydration processes, the concentration of RNA outside the liposomes was determined immediately after rehydration via the RiboGreen assay (see Section Analysis of Encapsulated RNA upon

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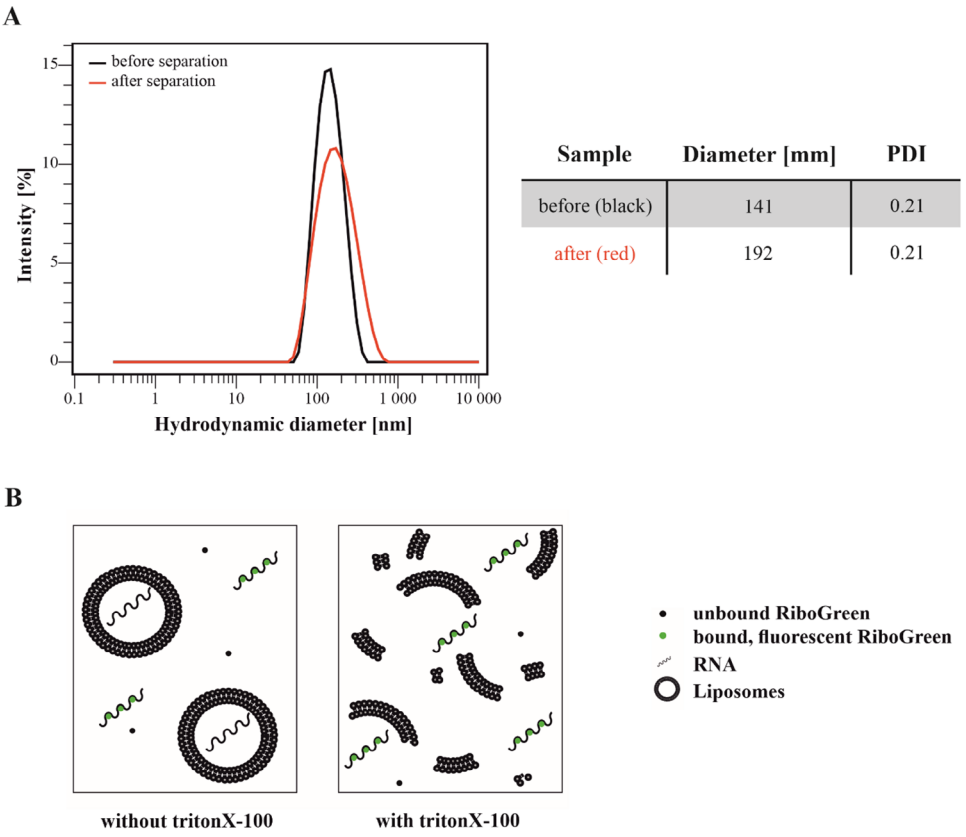
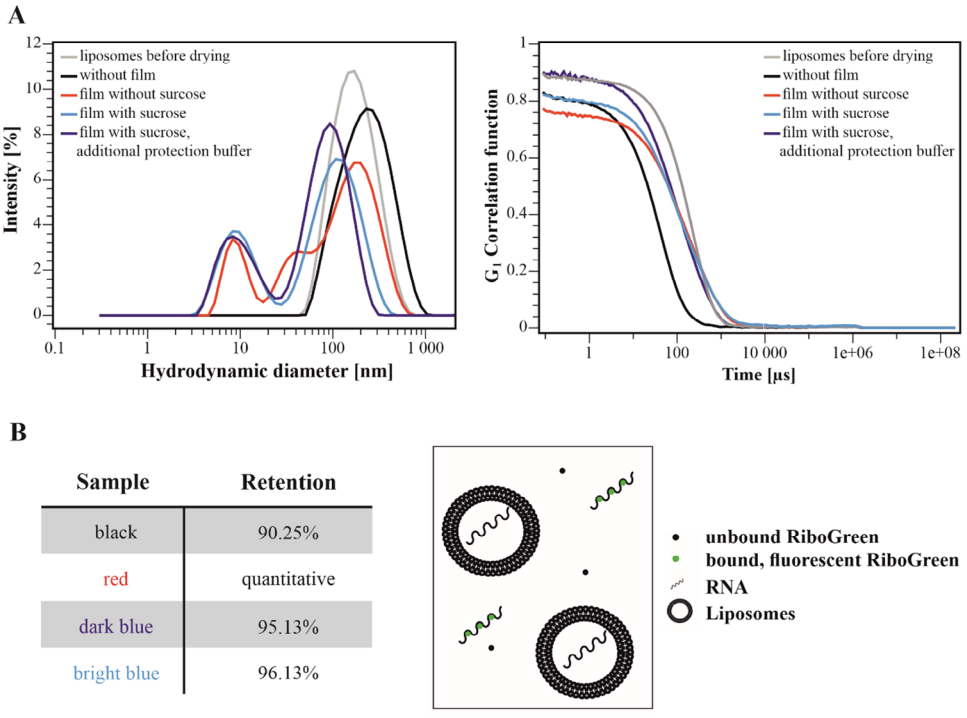


Sample	Diameter [mm]	PDI
before (black)	141	0.21
after (red)	192	0.21

Figure 3. Analysis of liposomal RNA encapsulation. A) DLS data showing the hydrodynamic diameter of the liposomes before (black curve) and after encapsulation of the RNA (red curve); B) Schematic representation of the RiboGreen assay for determination of the EE.



Sample	Retention
black	90.25%
red	quantitative
dark blue	95.13%
bright blue	96.13%

Figure 4. Analysis of liposomes loaded with RNA after application on the film, drying and rehydration. A) DLS measurements and correlation curves of dried and rehydrated liposomes under varying conditions. B) Calculated retention of the RNA in liposomes and schematic presentation of the RiboGreen assay used for analysis of RNA retention.

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Drying on the Film) (Figure 4B) and put in relation to the entire encapsulated RNA. In all samples the retention of RNA in the liposomes was higher than 90% (Figure 4B) with small variation from quantitative (liposomes applied on film without sucrose), to 90.25% (liposomes dried and rehydrated in the absence of the film as control), 95.13% (liposomes applied on film containing 250 mM sucrose) and 96.13% (liposomes applied on the film containing 250 mM sucrose and additionally being diluted 1:1 with sucrose containing buffer). Note that all liposome samples contained sucrose due to the preparation procedure (see 2.4). Storage of the dried samples on the EsoCap film did not show different results, the retention and the size of the liposomes did not change drastically.

3. Discussion

The application of therapeutic RNA on EsoCap compatible mucoadhesive films has the potential to treat esophagus diseases by targeting mRNAs encoding proteins that are upregulated in the course of the disease and have pathological effects on the cells and tissues. Therefore, we have tested the stability of free (naked) RNA and RNA encapsulated in liposomes on the film. As expected, the experiments with free RNA demonstrated that there was no degradative effect of the EsoCap film constituents. The free RNA, in non-labelled or fluorescently labeled fashion remained stable after subjection onto the film, drying, storage and rehydration. No difference was observed when the RNA was applied to the dry or wet film. In any case, RNA was transferred onto the film in a buffered solution where the RNA was dissolved in a molecularly dispersed solution. Given the small size of the RNA particles in comparison to the polymer particles, no change in surface properties was to be expected and could not be visually detected. After the application of the RNA solution onto the dry film and additional drying, the shape of the droplet was visually detectable. This is due to the characteristics of the film-forming polymer. The polymer is water-soluble, so the addition of liquid to the already dried film resulted in the dissolution of polymer particles, which then dried again, leaving a drop-sized shape on the film. Furthermore, the loading of films with active ingredients can have an influence on the mechanical stability of the film. Due to the usually low loading, which is sufficient when using highly potent active ingredients, no change in the mechanical properties of the film is to be expected through the application of the RNA. Thin, 4 mm wide film strips made of polyvinyl alcohol with a thickness of ≈100 µm and a water content that is in equilibrium with the room humidity easily exhibit a tensile strength of up to 10 N.

Liposomes being superior vehicles for RNA transport into cells and providing protection against nucleases were also prepared, transferred onto the film, dried, rehydrated and analyzed with a focus on liposome integrity and RNA retention.[19] When preparing the liposomes, their initial mean size, as measured by DLS, was 141 nm. After encapsulation of the RNA and separation of liposomes from free RNA, the mean size increased to 192 nm. This increase is possibly a result of the use of ethanol during the encapsulation step, as ethanol is known to increase liposome size, because the lipid bilayer is prone to form interlocking structures making liposome aggregation more likely.[20] To prevent excessive increase of liposome size, we performed a dialysis immediately after addition of ethanol. Also, the presence of calcium ions can affect the liposome size, although its impact is typically observed only over longer storage times.[21] Calcium ions are required to neutralize the negative charges at the phosphate backbone and are superior over magnesium or manganese ions, because of their easier removal.[22] The desirable size of liposomes for drug delivery applications ranges from 50 to 200 nm.[23] The observed sizes and the corresponding polydispersity indices (PDI) of the liposomes in our study were within well acceptable limits, ranging from 100 to 200 nm in size and having a PDI below 0.3, when measured without the EsoCap compatible film. With a PDI below the threshold of 0.3, one can speak of a virtually homogeneous liposome solution. In the presence of the EsoCap film, the PDI exceeded 0.5, due to the existence of both liposomes and the polymer PVA, resulting in non-homogeneous solutions. Nevertheless, the smooth autocorrelation curves observed also in these samples indicate high-quality and reliable measurements.[24] The RNA EE was, with 10.7%, lower than that reported by Somiya et al.,[18] but was considered to be sufficient for the further analytical part. A major influence of sucrose on the EE was not observed.

The variation in size observed when liposomes were dried after application on a still moist film in the absence of sucrose can be explained by lowering of the local sucrose concentration in the liposomes upon subjection on the watery surface of the film. Sucrose plays an important role in maintaining size stability during the drying process by creating a viscous environment that acts as a barrier between liposomes.[17] The control experiment, where liposomes were dried without prior subjection onto the EsoCap film, showed a slight increase of the liposome diameter after rehydration. This was as expected, as a slight increase of liposome size after storage for 1 h at 10 °C was reported also by others.[25] Liposomes dried on the film showed a slight size reduction compared to the state before drying. For preventing the fusion of liposomes, vitrification, being the full or partial transformation into a non-crystalline amorphous glass state, is very important and can be achieved by a high viscosity of the surrounding solution.[17] In addition to sucrose, which increases the viscosity of the sample, the film polymer itself also contributes to viscosity. Apparently, the polymer acts as a kind of barrier between the liposomes, such that fusion is prevented. The film consists of PVA with many hydroxy-groups, which can interact with the phosphates, as well as with sucrose and the lipid head groups. Furthermore, due to their strong water absorbing property, PVA and glycerol compete with the liposomes for the interaction with water during the rehydration process and thus assist in minimizing liposome leakage.[26] For all liposome samples good retention of the RNA was found, partially even higher than reported in the literature.[16] For preventing the leakage of the encapsulated drug, it is important to replace the water molecules and minimize the interaction between water and lipids.[17] Typically, sucrose or other disaccharides are used for this purpose.[16,17,25] We have chosen sucrose and observed the desired effects. The improved retention in samples that were subjected onto the EsoCap film in comparison to the controls in solution can be attributed to the additional support provided by PVA to minimizing interactions between water and lipids.

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Taken together, the EsoCap film is not only a suitable carrier for free RNA, but also for RNA encapsulated in liposomes to be applied to the esophagus.

Our research provides the foundation for future investigation into RNA therapies for esophagus diseases. This includes the definition of suitable targets, release of the RNA to the esophagus mucosa, cell penetration and functional response. If RNA based reduction or inhibition of proteins involved in esophagus pathologies can be achieved, application of those RNA drugs with the EsoCap system will offer an important alternative for therapy.

4. Conclusion

The present study demonstrates that RNA can be applied on the EsoCap system. The free RNA did not show any degradation upon subjection and drying on the polymer. In order to facilitate better transfection and protect the RNA against ubiquitous nucleases in the human body, liposomes were chosen as a transport vehicle and the integrity on the EsoCap film was investigated. Our results suggest that RNA encapsulated in liposomes can be used on the EsoCap system without the danger of degradation, RNA leakage or liposome fusion. This provides a promising perspective for innovative drug formulations on the EsoCap system and paves the way to treatment of esophagus pathologies with RNA or oligonucleotide-based therapeutics.

5. Experimental Section

Materials: 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and the instruments for the liposome preparation, extruder set, and polycarbonate membranes with a 100 nm pore size, were purchased from Avanti Polar Lipids inc. (Alabaster, USA). The tested oligonucleotides with and without ATTO633 label (5′-AUUUCGAGUUGGCUGUUGCUU-3′ (RNA A) and 5′-AUUUCGAGUUGGCUGUUGCUU-ATTO633-3′ (RNA B)) were obtained in HPLC grade from biomers.net GmbH (Ulm, Germany). Ethylenediaminetetraacetic acid (EDTA), ethanol (99.9%) and potassium chloride were supplied by Merck KGaA (Darmstadt, Germany), acrylamide/bisacrylamide (19:1), ammonium persulfate, calcium chloride, sodium chloride, tetramethylethylenediamine and trichloromethane by Carl Roth GmbH (Karlsruhe, Germany), and disodium phosphate, formamide, sucrose and triethylammonium-bicarbonate by Sigma Aldrich (Taufkirchen, Germany). Tris(hydroxymethyl)aminomethane was purchased from Serva (Heidelberg, Germany) and Quant-it RiboGreen reagent from Invitrogen (Carlsbad, USA). Urea was supplied by J.T. Baker (New Jersey, USA). The gel staining solution SYBR gold was purchased from Thermo Fisher Scientific (Waltham, USA). For the film preparation pharmaceutical broad spectrum polyvinyl alcohol 18–88 (PVA), with a degree of hydrolysis of 88% (Emprove series) and viscosity of 18 mPa*s (4% solution at 20 °C in water), was kindly provided by Merck KGaA (Darmstadt, Germany). Glycerol water free was purchased from Caelo (Germany). TritonX-100 was supplied by Acris Feinchemikalien GmbH (Heidelberg, Germany), Sephadex G-25 and G-50 from Pharmacia (Uppsala, Sweden). Autoclave DX-200 2D was from Systec (Wettenberg, Germany), Laminar air-flow box MS2020 1.8 from Thermo Scientific (Langenselbold, Germany) and Photosystem iBright FL 1500 from Invitrogen (Carlsbad, USA). For measuring the fluorescence of the RiboGreen, a DS-11 spectrofluorimeter from Denovix (Wilmington, USA) was used. For liposome size determination by dynamic light scattering (DLS), the Zetasizer Ultra from Malvern instruments (Herrenberg, Germany) was used. The rheometer DV3T extra was from Brookfield (Firmware V. 1.2.2-9, Hadamar-Steinbach, Germany) and the thermostat for temperature control was a Julabo Labortechnik GmbH system (Seelbach, Germany). The refractometer RX-5000 α-Plus (Atago Co., LTD, Tokyo, Japan) was used to determine the refractive indices.

Preparation of the EsoCap Film: The solvent-casting evaporation method was used for film production.[27] In brief, the polymer film consists of polyvinyl alcohol (PVA) (18 g) type 18–88, glycerol (2 g). As solvent during the production process sterilized water (80 g) for injections was used. The components were mixed in a laboratory glass bottle, heated up to 80 °C in a water bath, and left at this temperature under constant stirring for 2 h. Thereafter, the mixture was left to cool down to room temperature at stirring overnight to produce a bubble-free viscous solution.

The bubble-free mass was then sterilized in an autoclave for 15 min at 121 °C according to the standard pharmacopoeia parameter procedure with a reference vessel for temperature control, and was placed into a laminar air-flow box after cooling for further aseptic work. All materials required for aseptic work were sterilized and disinfected before use and placed into the laminar air-flow box. Subsequently, the prepared polymer solution was manually and homogeneously distributed on a polyethylene release liner with the help of a square applicator (gap height: 500 µm). The casted polymer solution was then dried overnight under aseptic conditions in the laminar air-flow box.

The polymer, either still moist or dried, was cut into pieces of ≈1 cm^2 together with the liner. The prepared RNA samples were then subjected onto the film from an Eppendorf pipette. The prepared and loaded films were dried at room temperature overnight in the laminar air-flow box (free RNA) or for three hours under vacuum in a desiccator (liposomal encapsulated RNA), respectively. After the drying process, the film strips were put in reaction vessels and stored at 4 °C.

Analysis of Free RNA upon Drying on the Film: In the initial tests of determining the compatibility of RNA with an EsoCap system compatible film, a non-modified naked model RNA was subjected onto the film, either as non-labeled variant (RNA A) or conjugated at its 3′-terminus to ATTO633 (RNA B). After drying, the film was rehydrated with deionized water (100 µL). For analysis of the RNA, a 20% denaturing polyacrylamide (PAA) gel was prepared. 1 µL of the solution containing the rehydrated film and RNA (corresponding to 5 pmol RNA) were diluted with water to 5 µL. The same volume of loading buffer was added. Following a denaturation period of 2 min at 90 °C, the samples were subjected to electrophoresis for 3 h at a voltage of 140 V. RNA bands were visualized by the fluorescence signal emitted at 675 nm by the samples labeled with ATTO633 upon excitation with light of 630 nm wavelength. For the samples that did not contain the dye at the 3′-end (RNA A), visualization was achieved by staining the gel with SYBR gold for 20 min, followed by UV imaging at 260 nm.

Liposome Preparation: At the start of the liposome preparation, DOPC (66 µL) dissolved in trichloromethane (25 mg mL^{-1}) was introduced into a glass tube. The solution was then dried using a nitrogen stream, followed by an overnight drying process under vacuum. For rehydration, Tris-HCl buffer (pH 8) containing 250 mM sucrose was used. In the next step, unilamellar liposomes were produced by eight freeze and thaw cycles in liquid nitrogen and at 54 °C. Thereafter, liposomes were homogenized by the extrusion method.

Encapsulation of the RNA was achieved following the protocol of Somiya et al., where an RNA content higher than 10 wt.% was recommended.[18] Briefly, the buffered solution containing the preformed liposomes (150 µL) were added to dry RNA (7.5 nmol), such that the RNA content was 20 wt.%. During constant mixing, a solution of 10 mM $CaCl_2$ in ethanol was added dropwise. The end concentration of the lipids was 1 mg mL^{-1} and that of $CaCl_2$ 4 mM. Finally, the sample was dialyzed overnight at 4 °C, with a membrane cut-off of 1 kDa against liposome buffer (PBS pH 7.4 containing 250 mM sucrose).

For separation of the free RNA from the liposomes, magnetic beads with strong anion exchanger (SAX) on the surface were used. For this step the SAX stock solution (20 µL) were put in a reaction tube. After 2 min on a magnetic separator the supernatant was removed. The liposome buffer (100 µL) were added and incubated for 2 min. The mixture was given on the magnetic separator again und left there for another 2 min. Then, the buffer was removed and the liposomes were added to the reaction tube. Resuspension was achieved by eight times up and down pipetting. After short incubation (30 s) the tube was put on the magnetic separator again for 2 min. The liposomes were removed and the whole process was repeated twice with 5 µL magnetic beads stock each time. Finally, liposomes

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were subjected to analysis. All used liposomes were stored in liposome buffer (PBS pH 7.4 containing 250 mM sucrose).

Determination of Encapsulation Efficiency (EE): For calculation of EE (Equation 1), first the RNA concentration in the samples with intact liposomes was determined using the RiboGreen reagent. Subsequently, the liposomes were disrupted with tritonX-100 and the total RNA content was determined.[28]

$$EE = 100 - \left(\frac{n_{(total)} - n_{(free)}}{n_{(encapsulated)}} \right) \quad (1)$$

For the first standard series, liposomes without RNA were diluted 1:500 with TE-buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 7.5). The desired amount of RNA was added to generate standards ranging from 0 to 75 nM end concentration. This solution (199 µL) were filled into the measuring tube, and a 1:200 with TE buffer diluted commercial RiboGreen reagent solution (in DMSO) (100 µL) was added. All samples to be measured were equally treated. After mixing and incubation for 3 min in the absence of light, the samples were irradiated with light at a wavelength of 480 nm, and the fluorescence at 525 nm was measured. For determination of the total RNA content, the samples and liposomes for another standard series were diluted 1:500 with TE-buffer containing 2% tritonX-100. liposomes were disrupted by tritonX-100 and the encapsulated RNA was released. All following steps were carried out as described above for intact liposomes. The amount of free RNA (non-encapsulated) in relation to total RNA was determined and the EE was calculated using Equation (1).

Liposome Homogeneity and Sizing: The size and homogeneity of the liposomes were determined by dynamic light scattering (DLS). The fully prepared samples, after the separation of free RNA by magnetic beads, were diluted 1:100 with deionized water. This solution (100 µL) were transferred into a micro cuvette with a path length of 10 mm. After an equilibration time of 2 min at 25 °C the scattering was measured in an angle of 173°. Parameters were chosen according to the DLS manual, being viscosity of 0.833 mPa*s, absorption of 0.001 and refractive index of 1.33, for aqueous solutions.

Analysis of Encapsulated RNA upon Drying on the Film: For analysis of the encapsulated RNA with the EsoCap system, samples (10 µL) were subjected and dried on the polymer film. Subsequently, the film was rehydrated with deionized water (100 µL). Thereafter, the size of the liposomes was determined in the same way as described in Section Liposome Homogeneity and Sizing. The only divergence in the settings for the measurement was the viscosity, which was determined to be 2.45 mPa*s at a temperature of 37 °C determined with the rheometer. The refractive index was also determined, because owing to the presence of the PVA-film in the sample, a divergence to pure water may be expected. Therefore, 1 cm^2 of pure EsoCap film (without RNA) was dissolved in deionized water (100 µL). One drop of the sample was placed in the refractometer and measured at a temperature of 20 °C. A refractive index of 1.33 was determined, being virtually the same as for pure water. All other instrument settings were identical to the measurements described in Section Liposome Homogeneity and Sizing. For measurement, the samples were diluted 1:10 with water. For analysis of RNA retention in the liposomes after drying for 3 h and rehydration, the concentration of the RNA outside the liposomes was measured using the RiboGreen reagent. The standard series was prepared as described in Section Analysis of Free RNA upon Drying on the Film, however, now using liposomes that were dried on the film and then rehydrated before use. Final RNA concentrations in the standard solutions were in the range from 0 to 7.5 nM. Because of the expected lower RNA concentrations, the RiboGreen reagent was diluted 1:2000.

Statistical Analysis: Experiments requiring statistical analysis were not performed.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

drugs, EsoCap, esophagus, liposomes, RNA

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