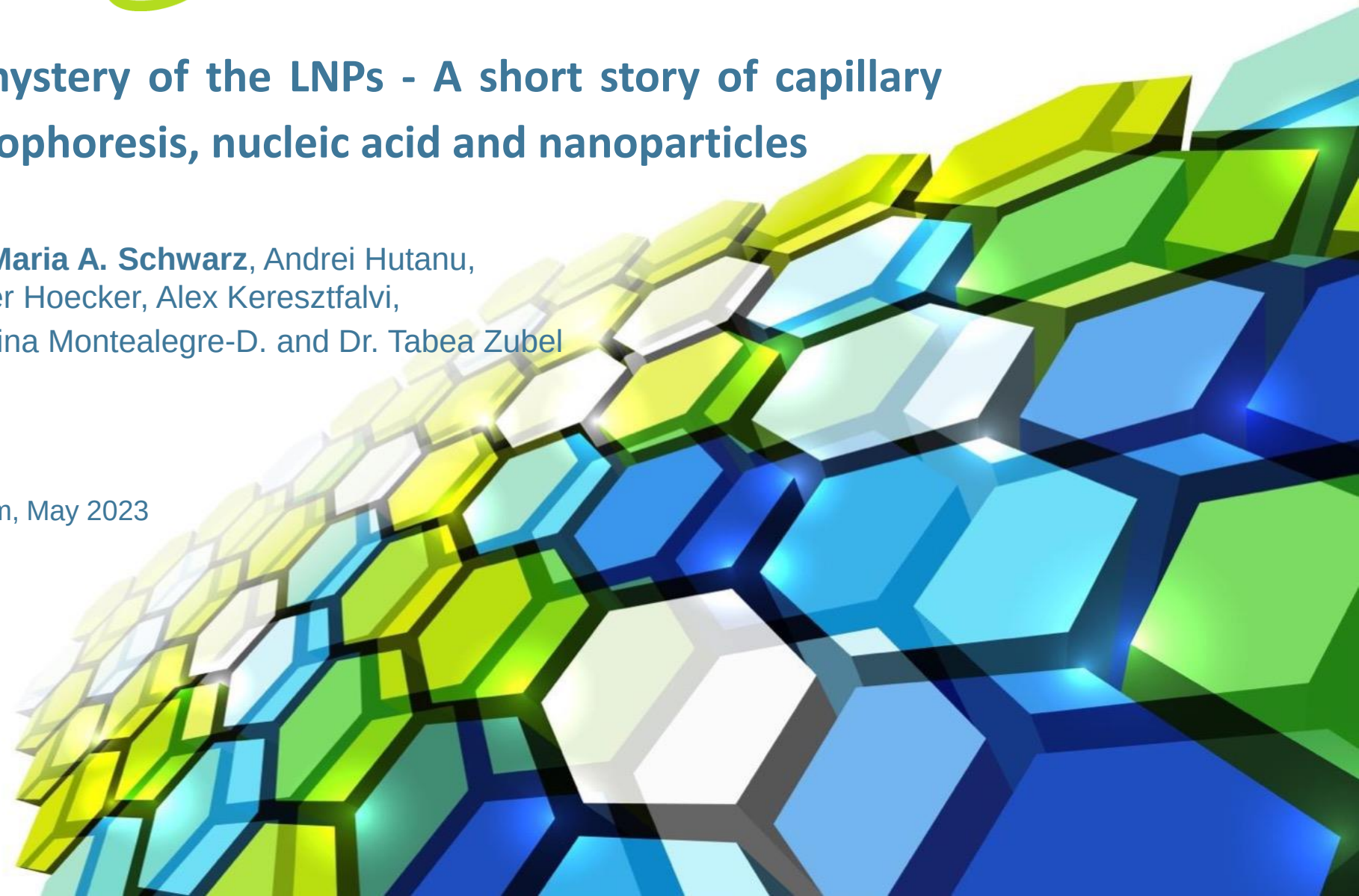
The mystery of the LNPs - A short story of capillary electrophoresis, nucleic acid and nanoparticles

PD Dr. Maria A. Schwarz, Andrei Hutanu,
Dr. Oliver Hoecker, Alex Keresztfalvi,
Dr. Cristina Montealegre-D. and Dr. Tabea Zubel

Rotterdam, May 2023



Abstract

As can be read consistently in various professional journals, nucleic acids (NAs) will play an increasingly important role as pharmaceutical therapeut of the therapeutic proteins. And as so often, adequate analytics is at the end of a development process, howe les enter the market robust analytics is not fit for purpose. So, it is time to tackle the new challenges even though a good foundation was laid in the pioneering days of NA analytics. The challenge is due to the fact that NAs occur in innumerable variants, as double strand or just single strand, in a mass range of 20 to 10000 b(p) meani MDa range, sometimes modified and encapsulated in nanoparticles (LNF

Most physicochemical techniques used for the analysis of therapeutic proteins are also applicable for nucleic acids. However, NAs show some limitations due to the i actions resulting in complex conformational and charge interaction behaviors. After a technical overview showing a strategy how NA analyt on to the focus of more complex LNP/RNA formulations. Both the quality/homogeneity of the LNP meaning free and encapsulated NA, are crucial analytical parameters. Helpful techniques for the characterization and evaluation of nanoheterogeneity of the LNP formulation are cryo-TEM and AF4, however either it needs a special technical knowledge or it is not w atory. Beside various HPLC methods for the characterization and quantification of released NA CE methods could be a grateful analytical tool for the clarification of both the localization/characterization of RNA as well as the evaluation of the LNP state.

Physicochemical techniques

Nucleic acids (NA)

Critical quality attributes

Characterization and quantification

Capillary electrophoresis

Charge of LNP

Encapsulation efficiency

Staining and hybridization

Localization RNA

Strategy for LNPs

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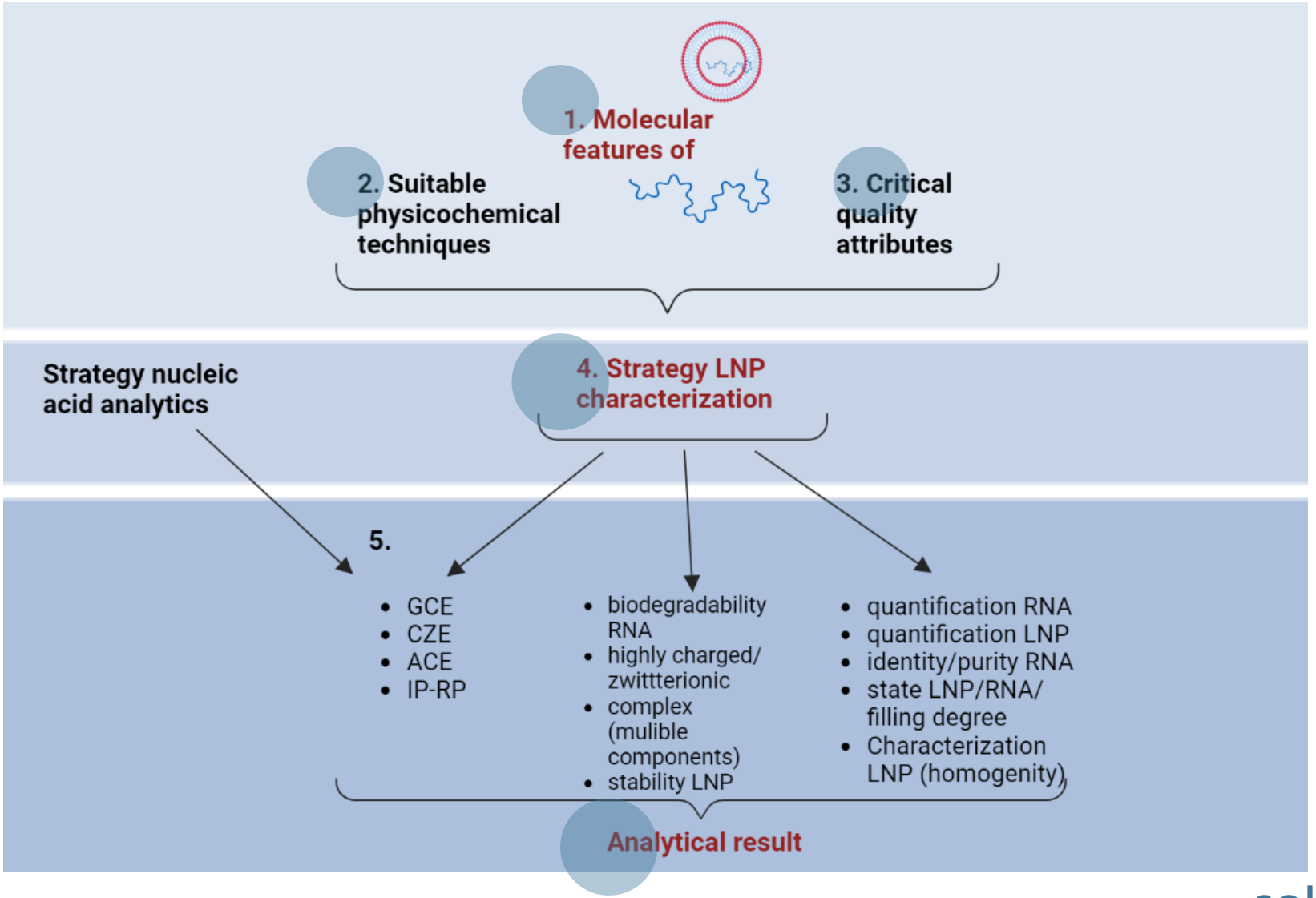
Overview



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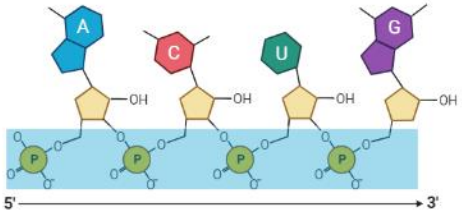
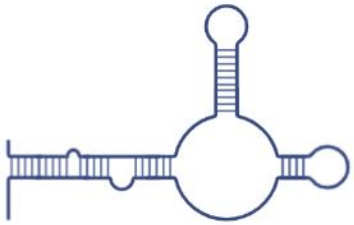
1. Molecular features

Single strand nucleic acids



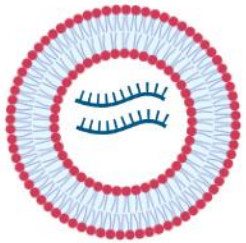
Backbone with strong negative charge



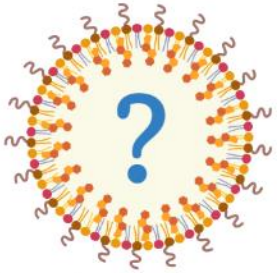
Inter- and intra-molecular hybridization

Highly susceptible to RNAse

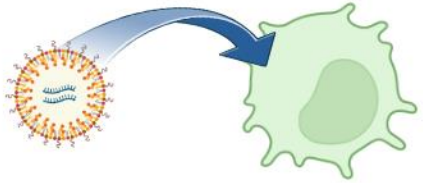
Lipid nano particles



Consists out of two phases



Actual morphology is unclear, but permeable with fluorescent dyes



Protects RNA and helps permeating target cells

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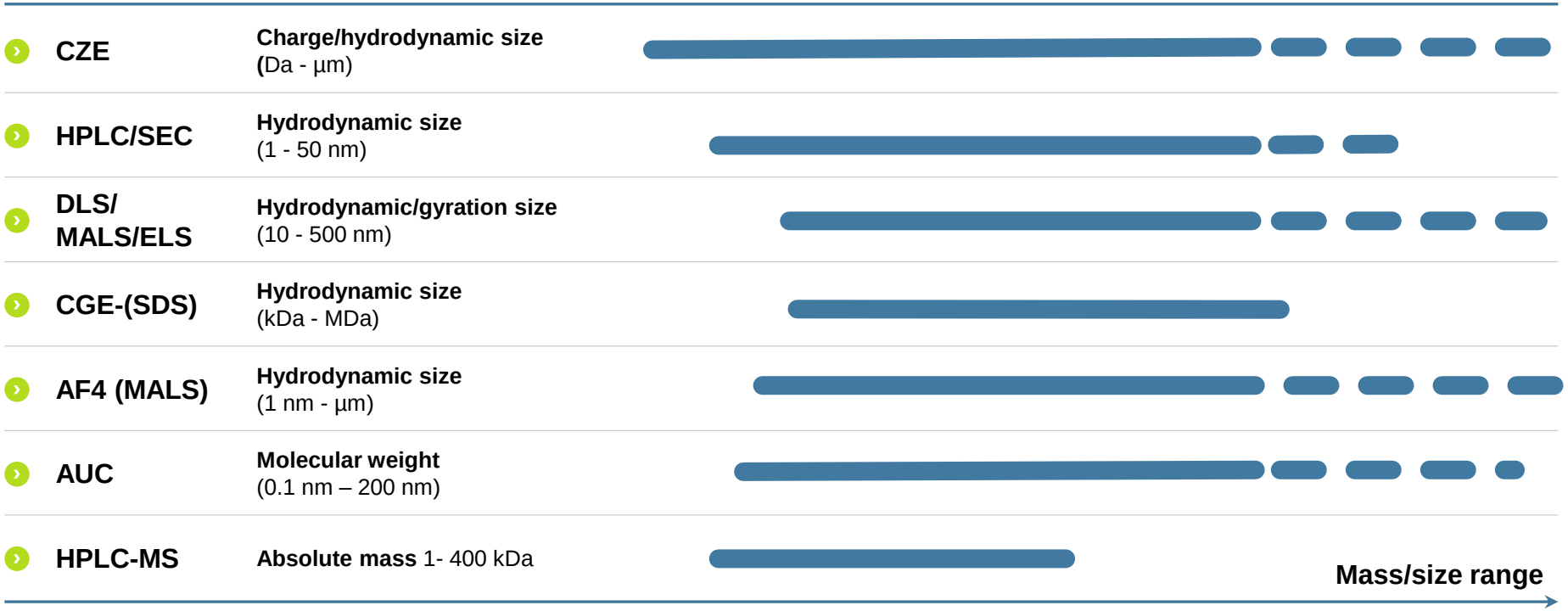
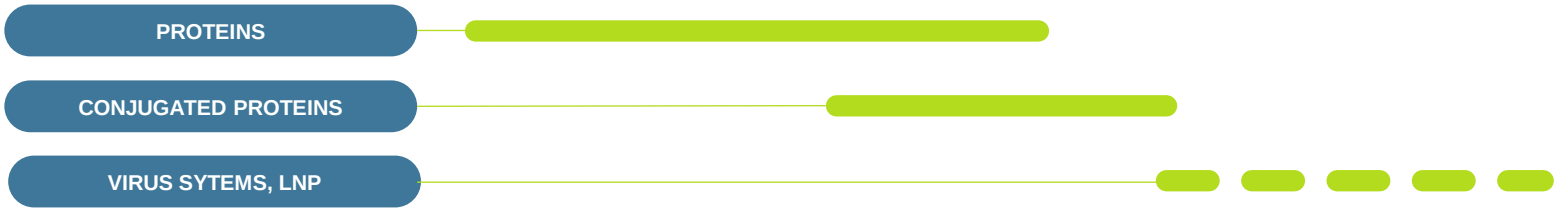
2. Physicochemical test methods suitable for large molecules and nano particles





2. Potential physicochemical test methods suitable for nucleic acid analysis

IP-RP-UV-MS	**Hydrophobicity based** Chemical modifications	MS
HILIC-UV-MS	**Hydrophobicity based** Chemical modifications	MS
CGE-UV/LIF	**Size based** Topoisoforms, size variants	
CZE-UV/LIF	**Charge/hydrodynamic size** Roughly quantification	
ACE-LIF	**Hybridization** Identity, quantification	
(q)PCR	**Enzyme based reproduction** Identity, quantification	
UV, FBA	**Direct UV, staining** Quantification	

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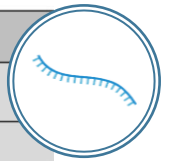
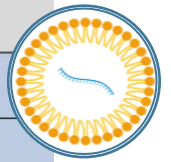
3. Critical quality attributes of LNP-RNA systems

CQAs	Potential test methods	Remarks
mRNA, DS		
Identity, sequence and quantification	Reversed transcriptase qPCR	Degradation products can only be quantified indirectly
Quantity	UV spectroscopy Fluorescence-based assay (FBA) ACE (affinity CE), IP-RP	UV/FBA: purity dependent
RNA related purity (identity and size)	GCE, IP-RP	Fragments and topoisoforms Identity is limited due to limited resolution in the high molecular mass range
Specific features as 5'end Capping efficiency 3' poly A tail length	GCE-LIF or IP-RP/HILIC	Specific primer needed
mRNA lipid complex, DP		
Quantity, localization RNA (encapsulation efficiency)	Fluorescence-based assay (FBA) IP-RP, ACE, CZE (free/total)	Limited specificity and dye permeation into LNP Suitable release of RNA, degradation of the LNP needed
RNA related purity (identity and size)	GCE, IP-RP	Suitable release of RNA, degradation of the LNP needed
Particle size, zeta potential,…	DLS, ELS and others Microscopy SEC/AF4-MALS	AF4 not very well established, high competency required
Intact LNP (identity, charge (distribution), **stability)**	CZE	Under development, not established





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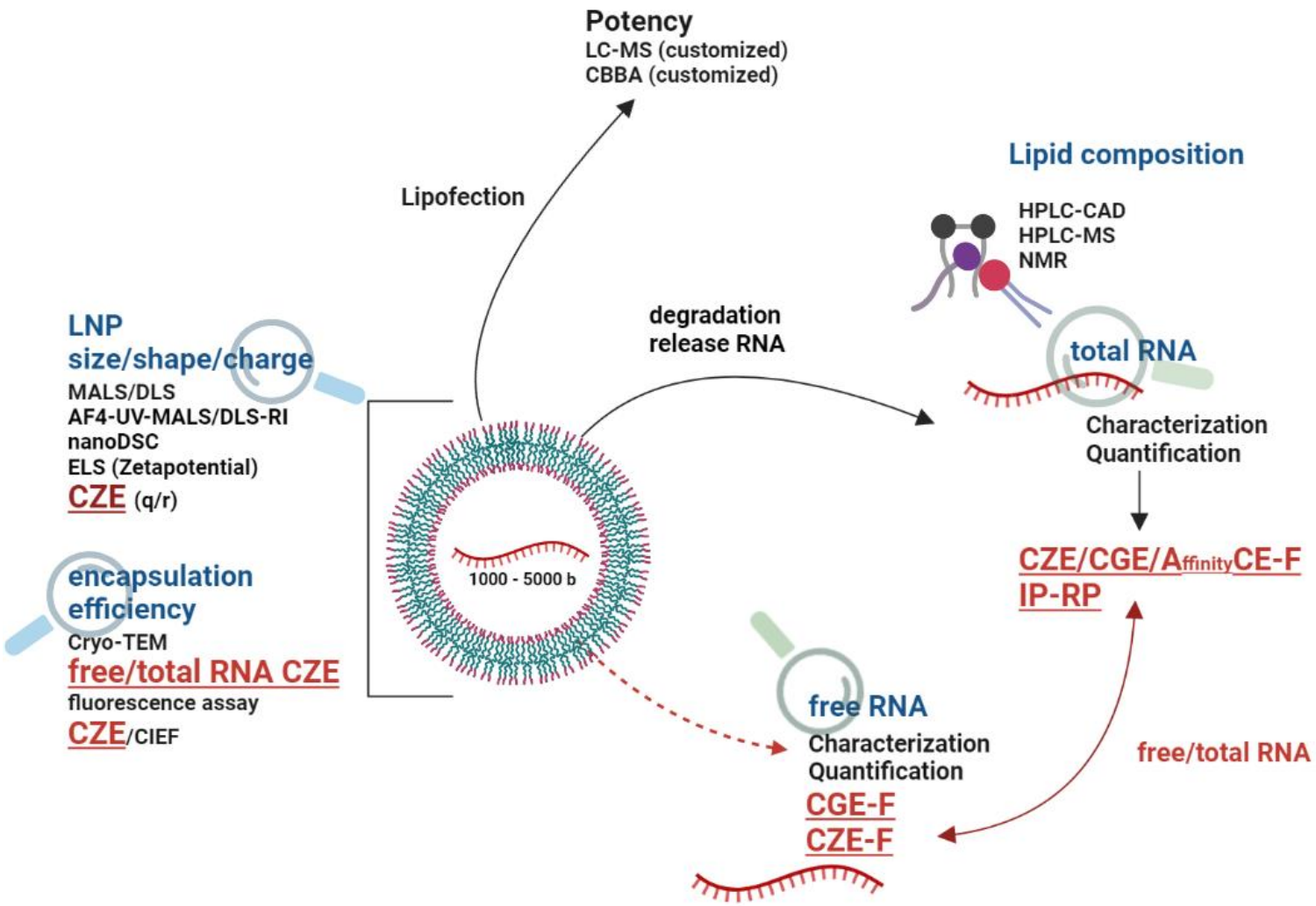
4. Strategy LNP characterization



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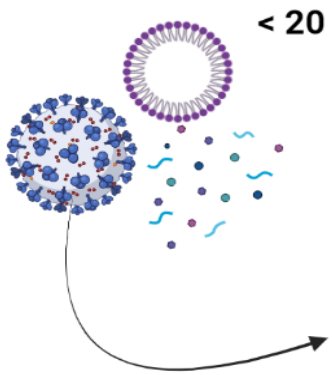
Why capillary electrophoresis?
Features und sub-modes



UV, LIF, others

- **Broad size range up to low µm**
- **Easy change of CE modes**
- **High sensitivity in case of LIF**

BGE

charge/rh — CZE/MEKC: native, + dye, + surfactants, + additives ✓

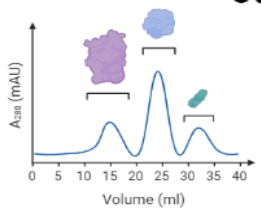
size — GCE (SDS): native (or denaturated), sieving matrix ✓

specific interaction — ACE: native, specific interacting receptor/molecule ✓

pI — CIEF: native, pH gardient **Only suitable for encapsulated NA** X

5 - 30 kV

Other controlling parameter: V, pH, ionic strength, EOF



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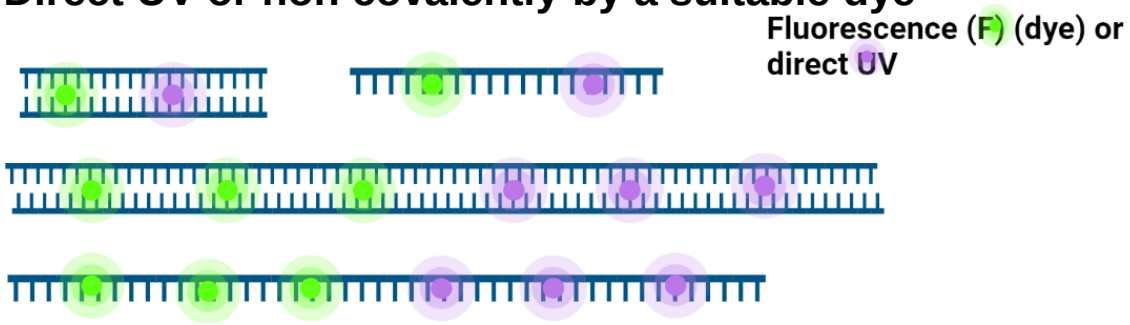
Features of detection

Direct UV or non covalently by a suitable dye



- Useful for NA **> 20 b(p) – 1 000 000 …..**
- Suitable for **ds and ss NA**
- Signal intensity = f **(number of base (pairs)**)
- Signal intensity = f **(ss or ds, tertiary structure)**
- Signal intensity = f **(A, G, T, C, U)**

For quantification purpose careful assessment of suitable reference

Hybridization by specific peptide nucleic acid

- Useful for NA **20 b – 6000 b**
- Suitable for **ss NA**
- **Sensitivity** = f (number of PNA hybrids)
- **Specificity** = f (length PNA and number of hybrids)
- Others: the presence of cD/RNA

NA sequence has to be known as well as strategy (number/length of PNA)

PNA – peptide based NA (affinity probe)

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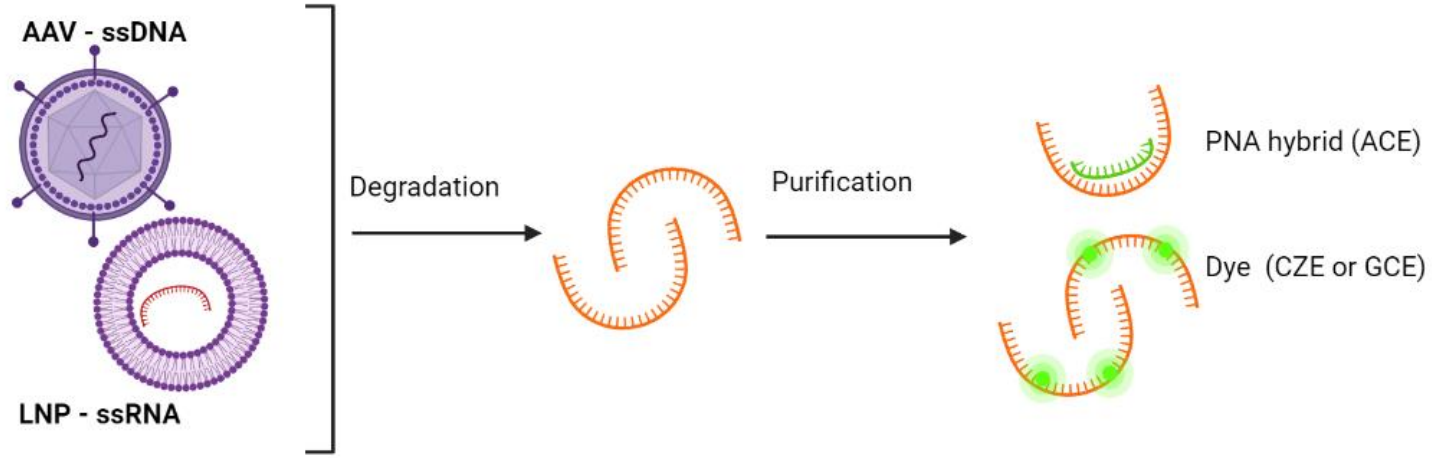
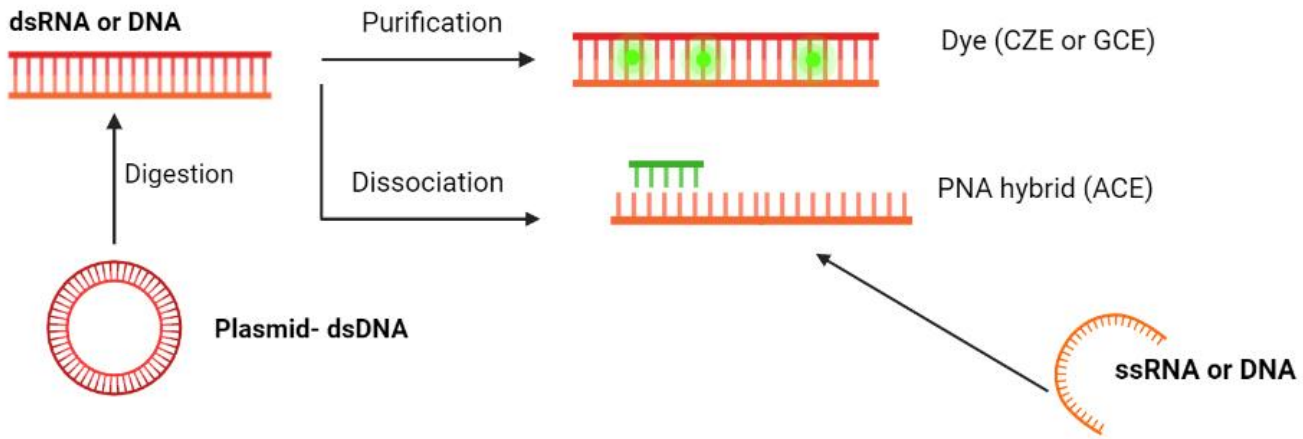
Encapsulated and free NA

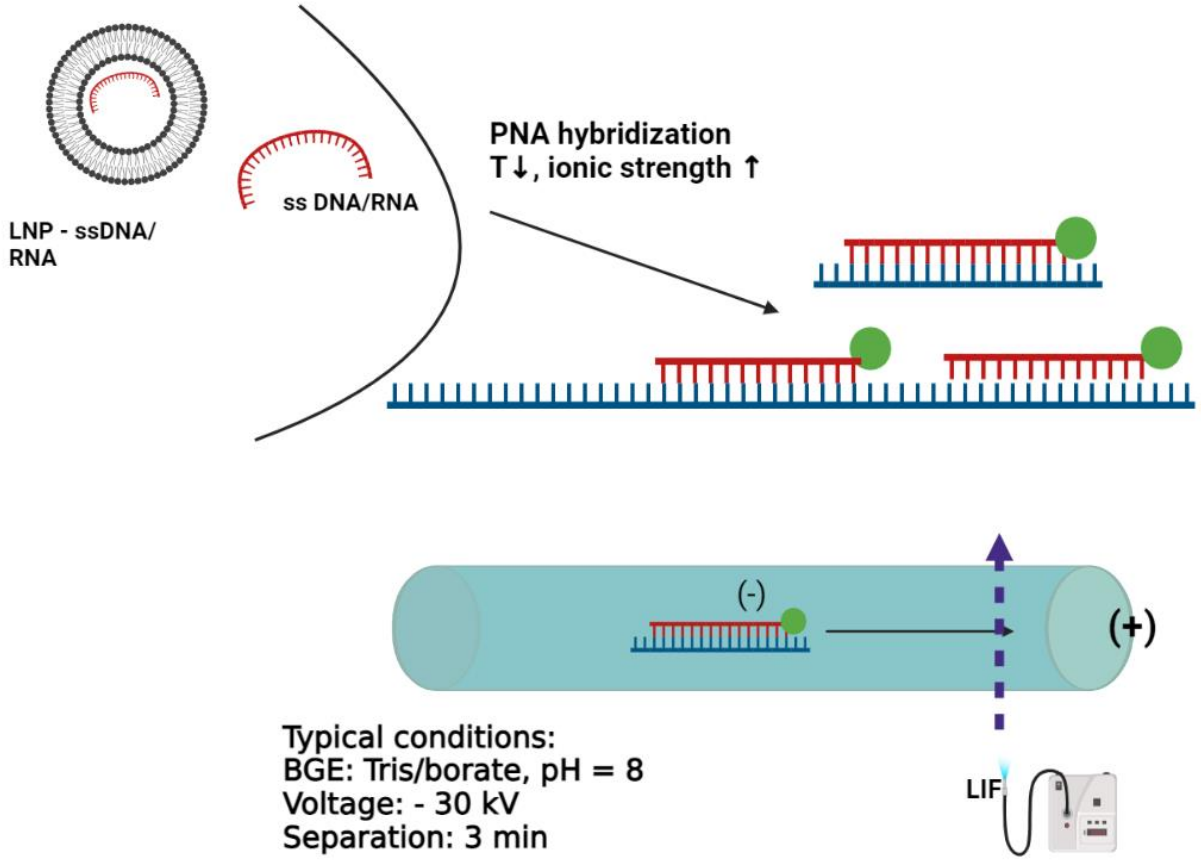
Encapsulated (R)NA



Free (R)NA



Principle AffinityCE



- **High selectivity** by hybridization with cPNA
- **Stable fluorescence signal** due to labelled PNA
- Not affected by **inter-/intramolecular interaction** or base stacking during preparation and separation
- Signal intensity **directly proportional** to the molarity of the target molecule (not size/mass dependent)

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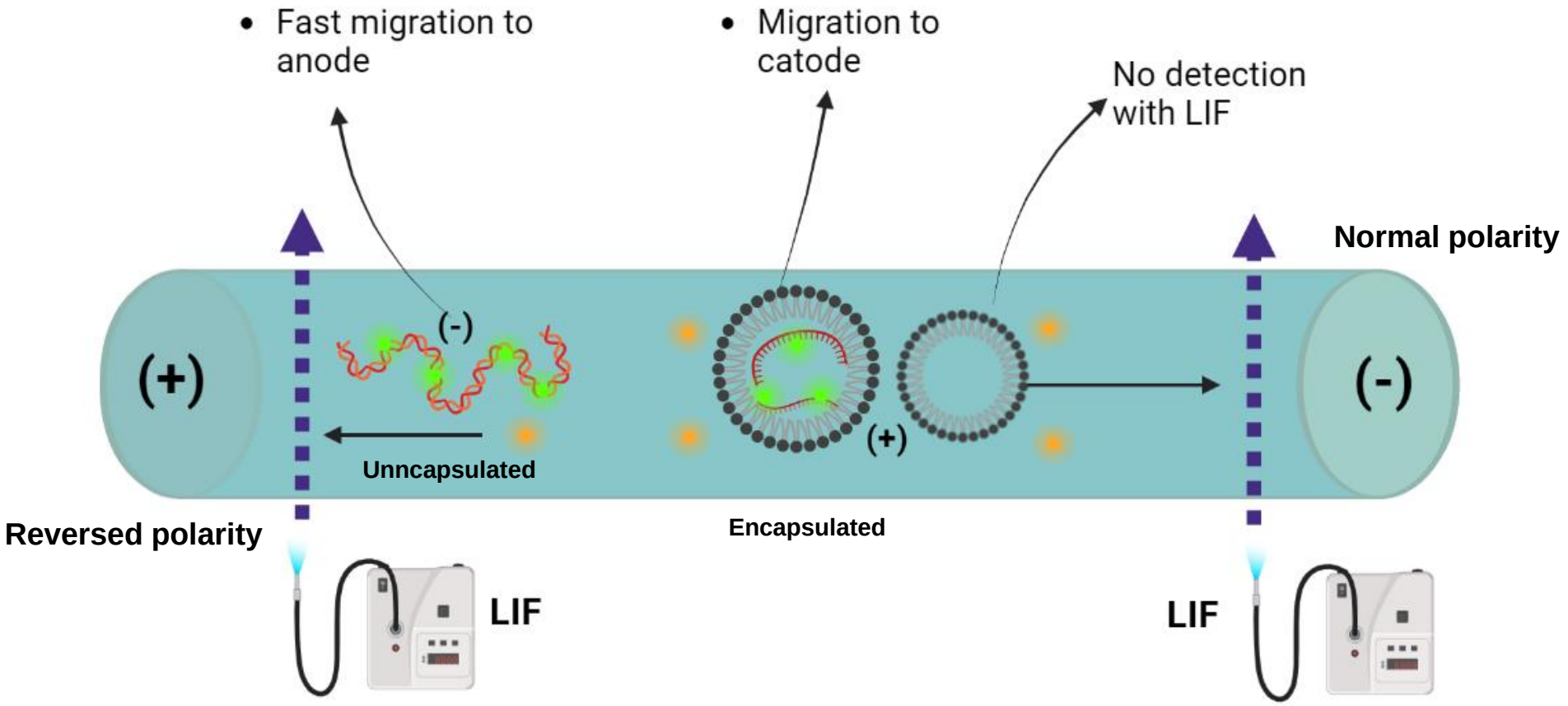
Principle of C(G)ZE of free and encapsulated NA



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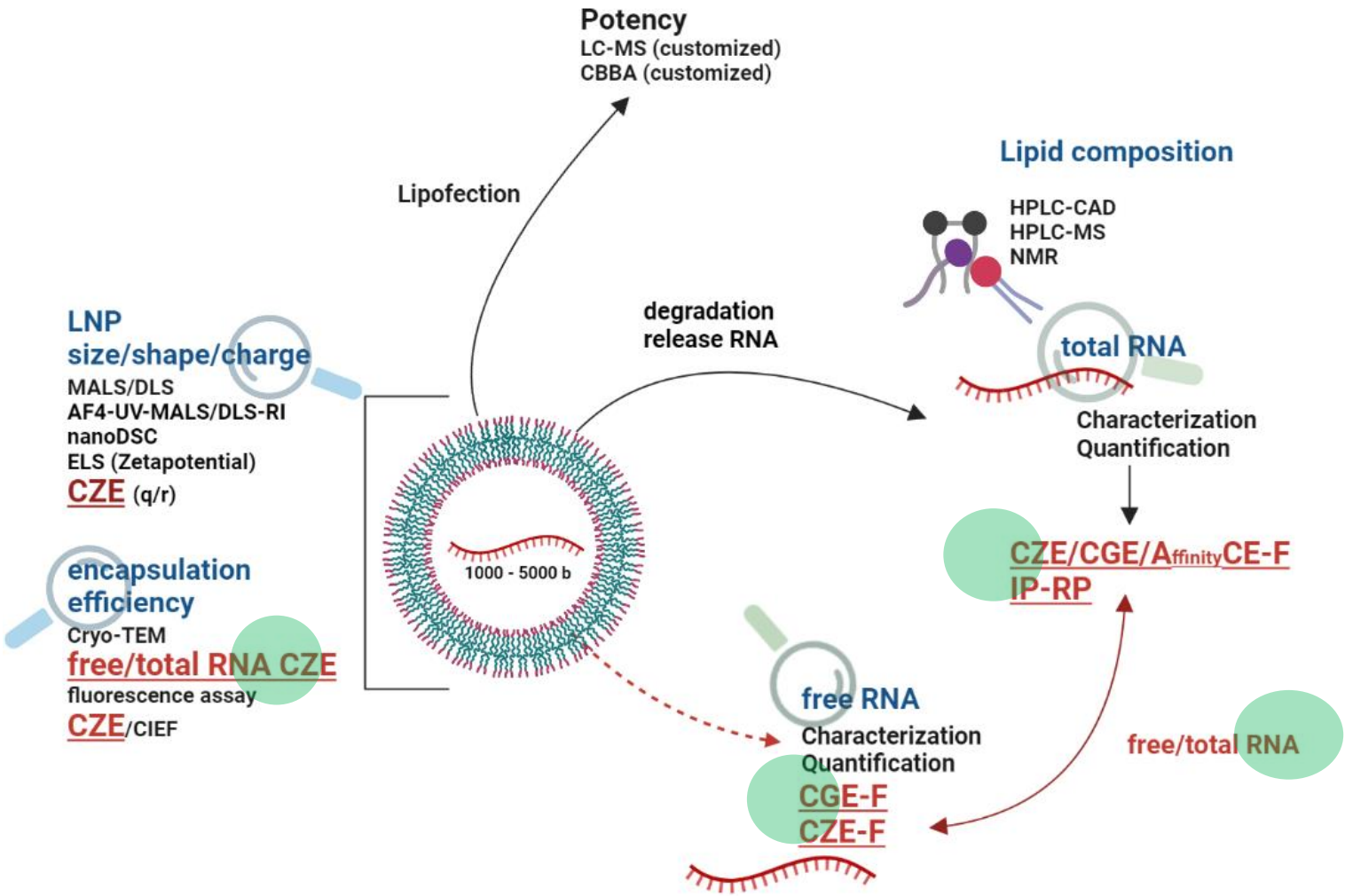
Strategy LNP characterization



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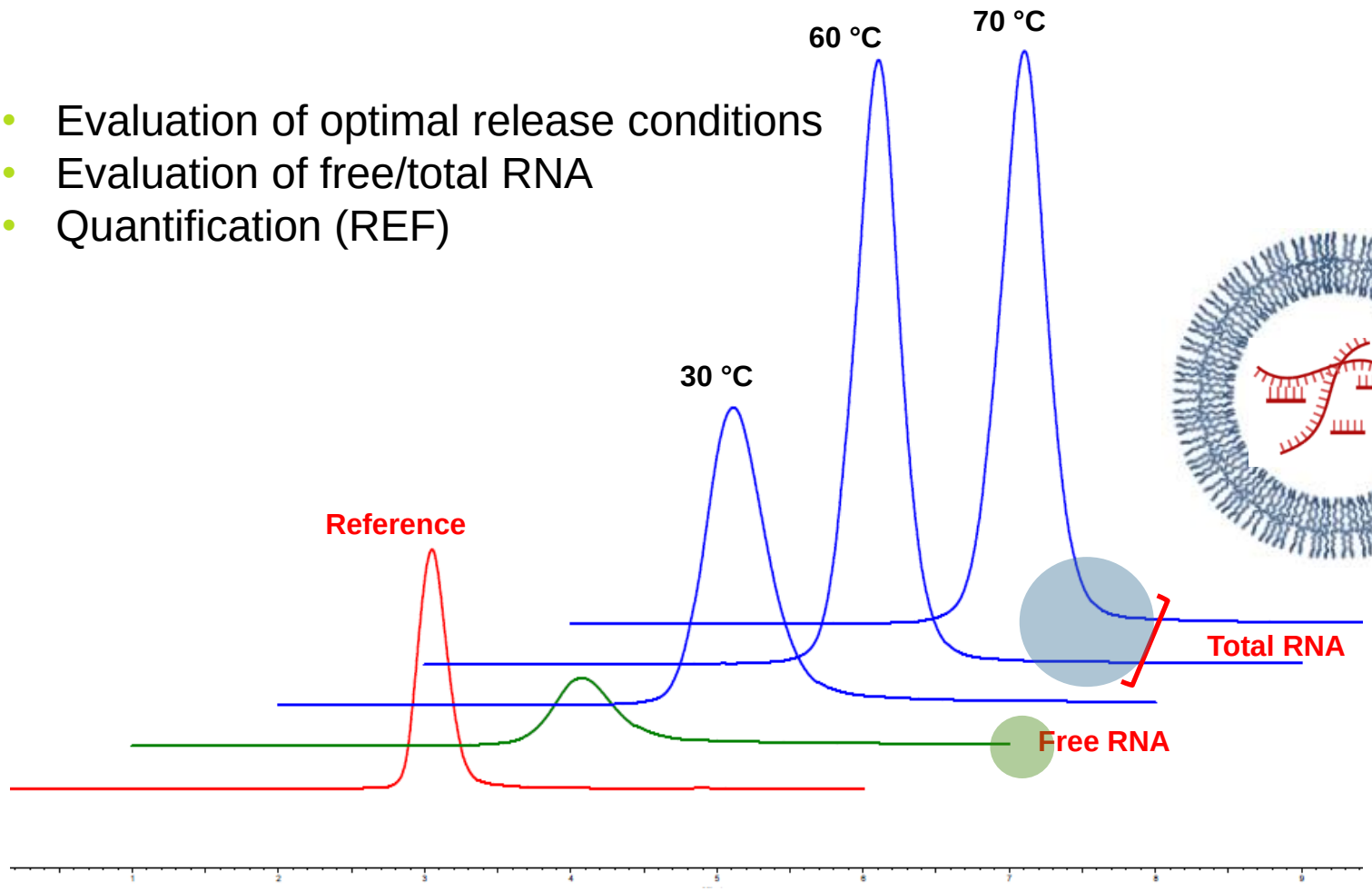
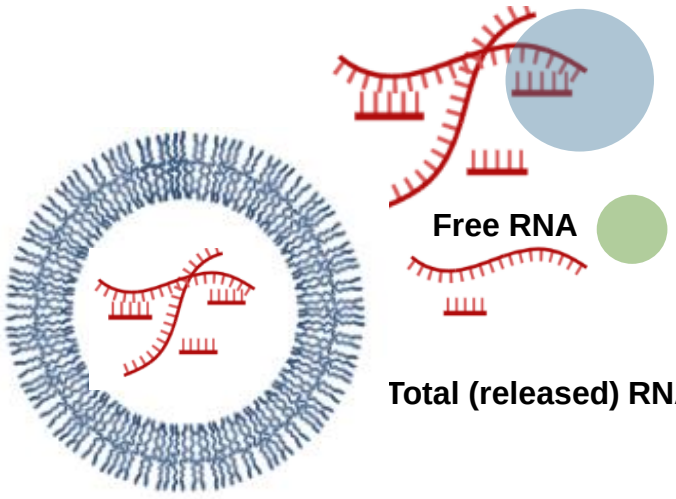
CZE: quantification free/total (released) RNA

- Evaluation of optimal release conditions
- Evaluation of free/total RNA
- Quantification (REF)



Incubation with non-ionic surfactants, T



c_{free}/c_{total} = 1/7

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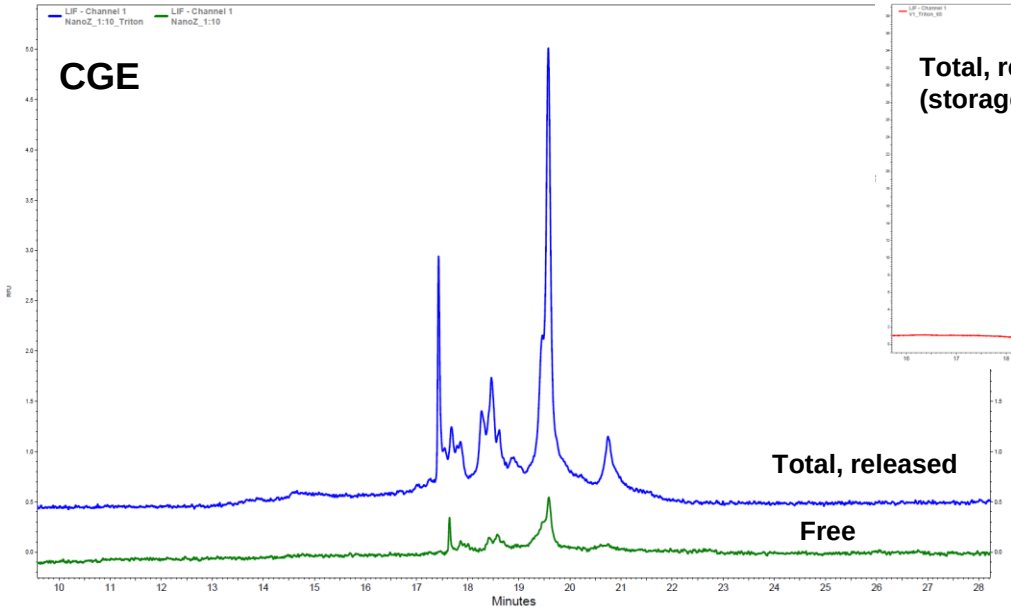
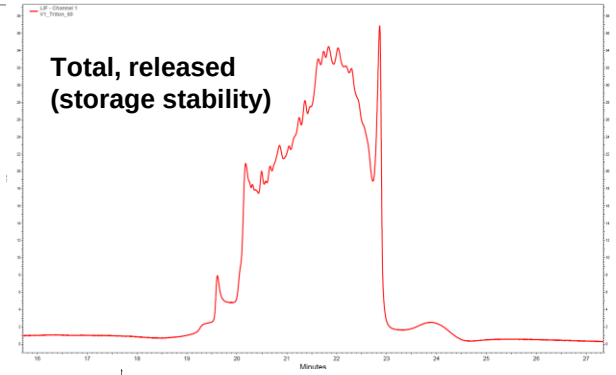
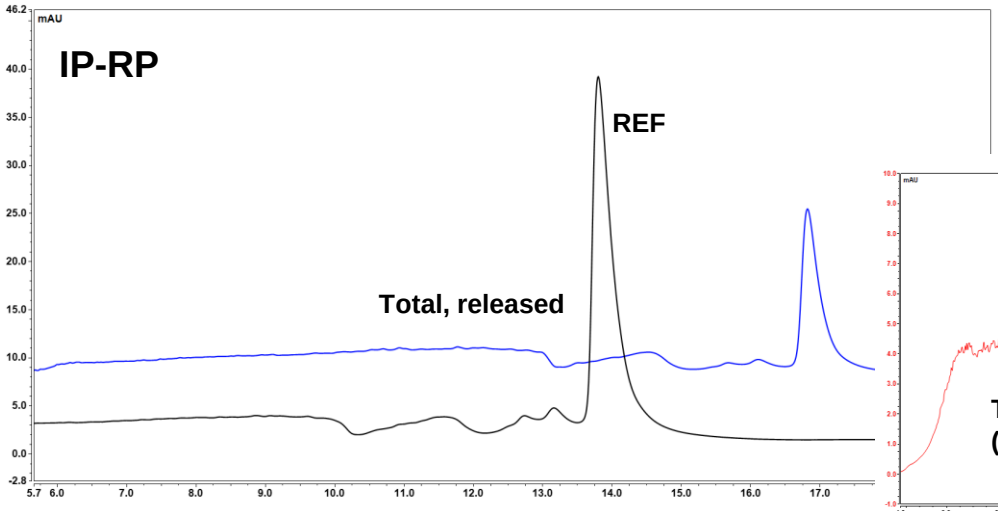
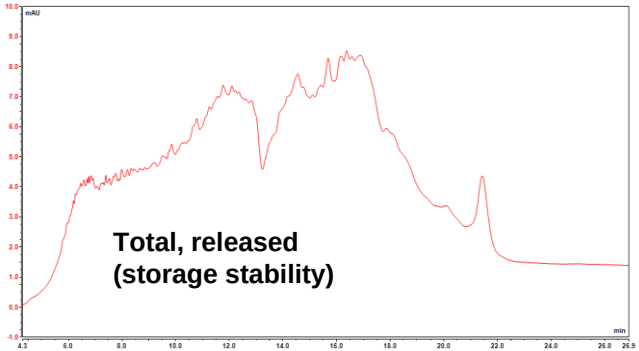
CGE and IP-RP: the characterization of free/total RNA



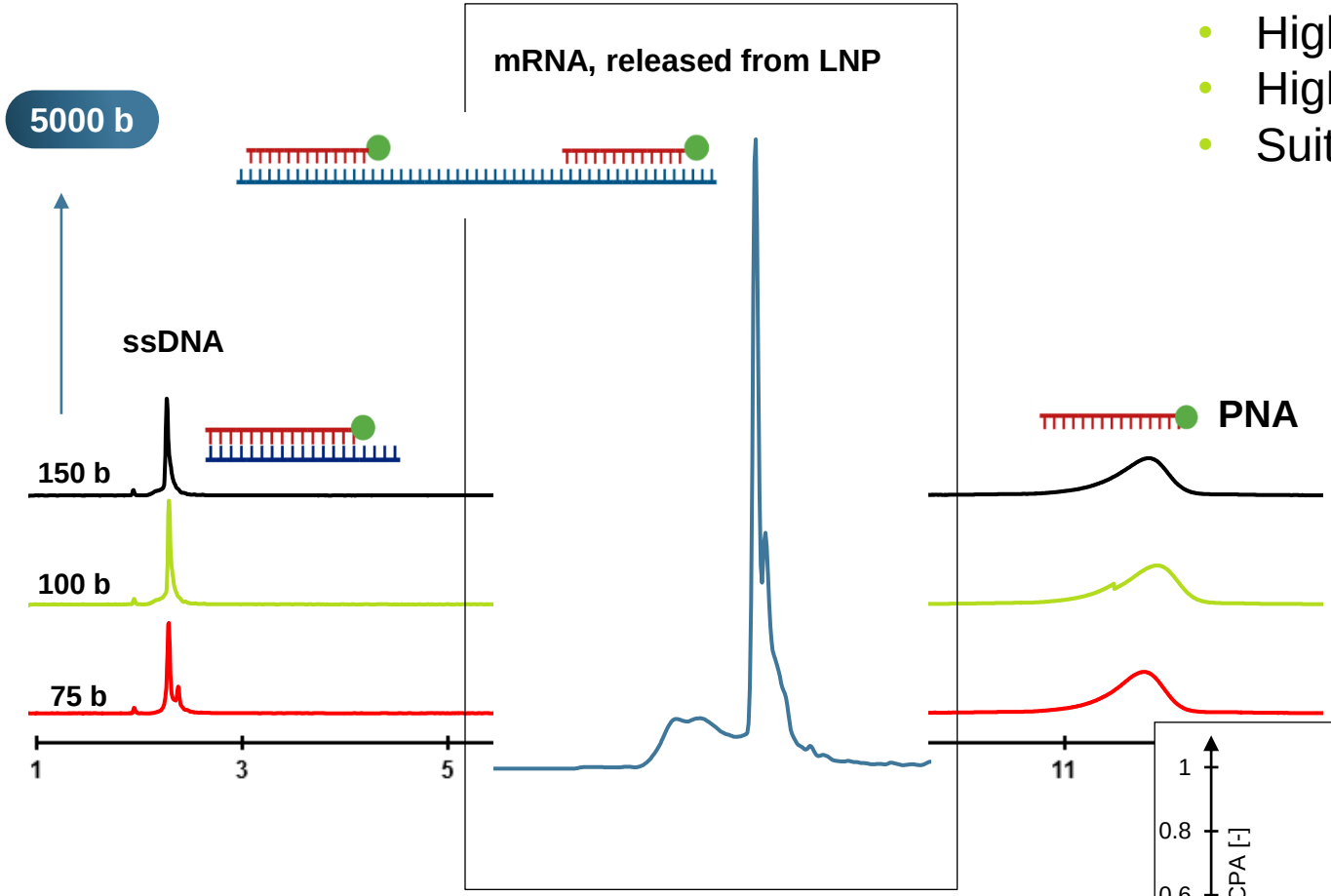


- Characterization of ‘free’ RNA by CGE (native)
- Release/total RNA profile are comparable
- The IP-RP profile of released RNA differs from GCE profile
- But not for strong degraded TS

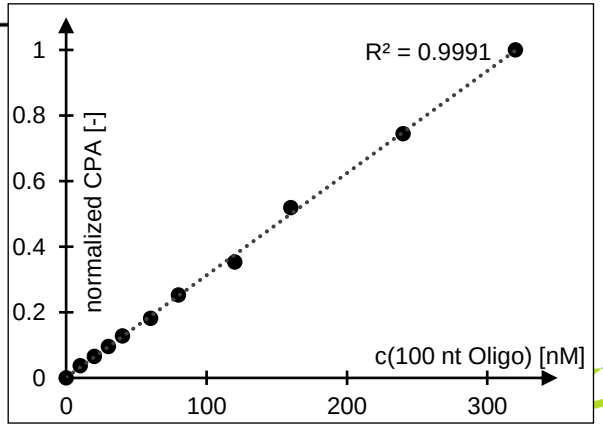




AffinityCE – from the implementation to the application



- Highly specific
- High sensitivity
- Suitable for LNP systems



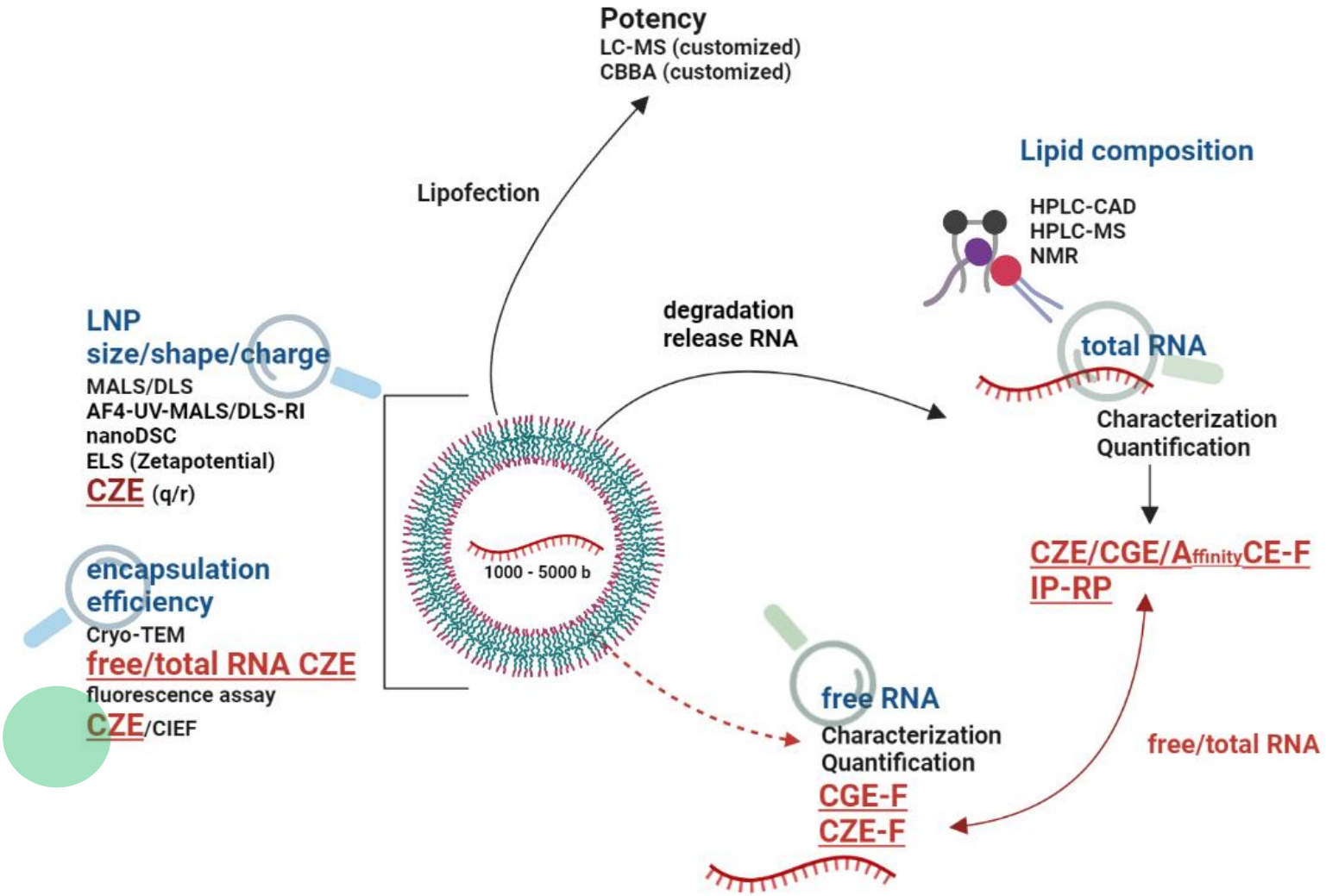
analytical chemistry

pubs.acs.org/ac Article

Using Peptide Nucleic Acid Hybridization Probes for Qualitative and Quantitative Analysis of Nucleic Acid Therapeutics by Capillary Electrophoresis

Andrei Hutanu,* Chiara Signori, Bernd Moritz, Manuel Gregoritza, Adelheid Rohde, and Maria A. Schwarz

Strategy LNP characterization



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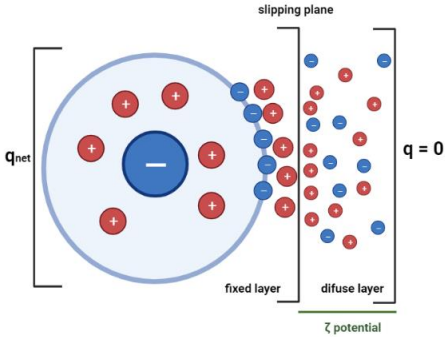
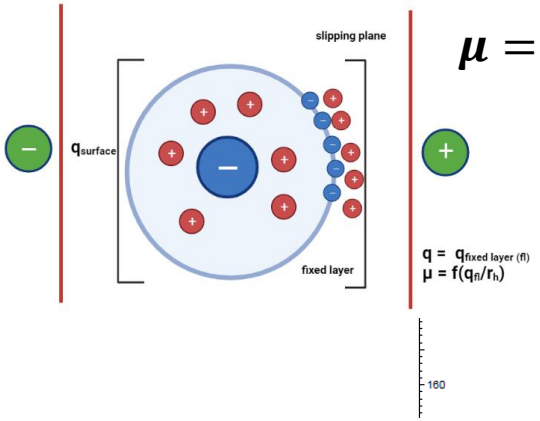
LNP characterization – charge, payload



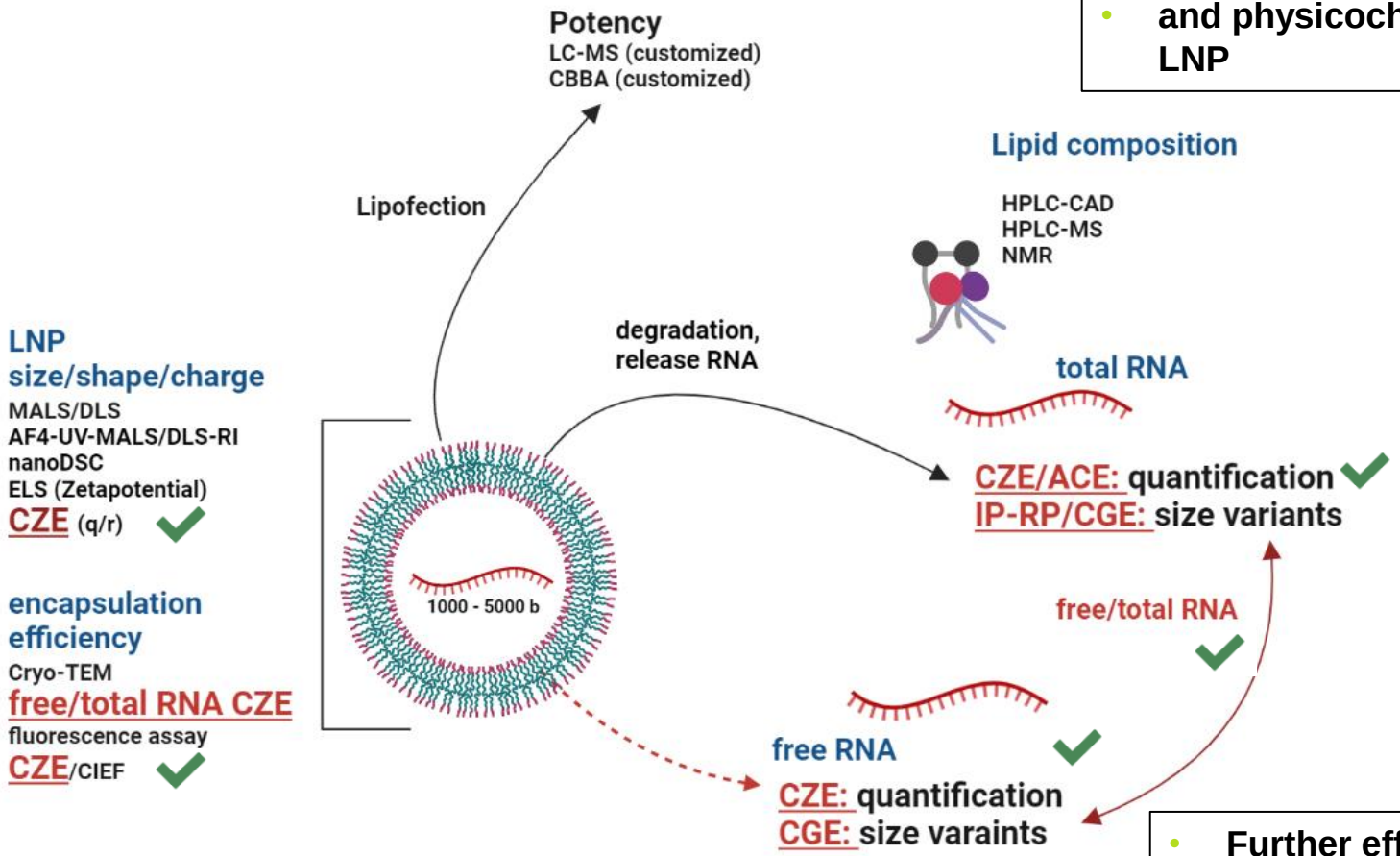


$$\mu = \frac{f\zeta}{\eta}$$

- Detection of the intact LNP and other variants
- visible by the RNA staining
- Intrinsic physicochemical features relevant
- for characterization and stability indicating features
- No complementary or orthogonal methods

Conclusion

- Where is the RNA localized,
- in which state,
- providing an encapsulation efficiency
- and physicochemical features of the intact LNP



- Further effort needed (higher order structure)
- Complementary techniques
- Meaningful and available material

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Hopefully, the mystery of the LNPs has been revealed a bit and there will probably be some more short stories to tell.

Thank you very much for your attention and .....

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Solvias AG

Römerpark 2, 4303 Kaiseraugst, Switzerland

www.solvias.com

Name, first.lastname@solvias.com, T +…