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Nové léčebné terapie a jejich analytika

Jak pandemie urychlila vývoj genových terapií

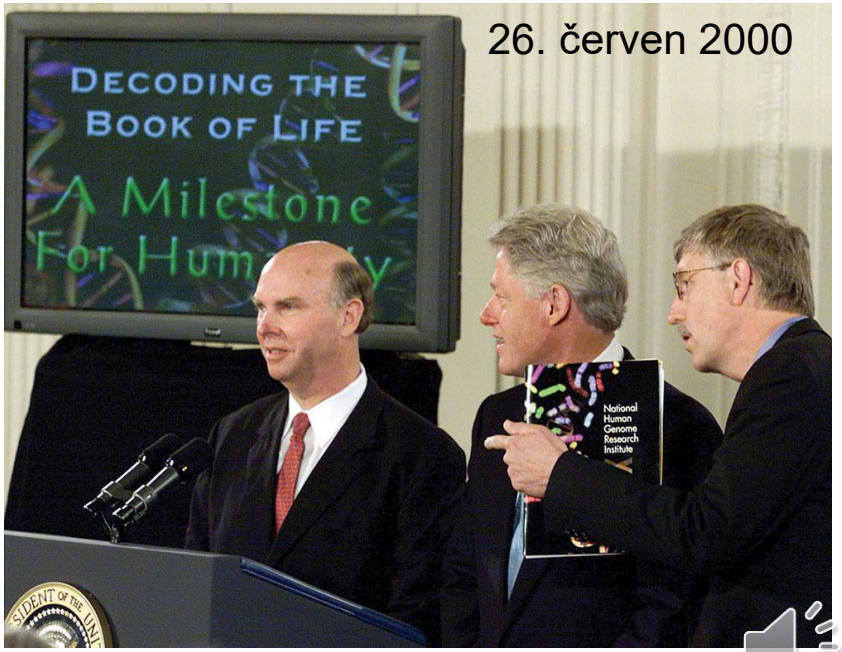
Martin Gilar

23. 11. 2021, Vize 2021

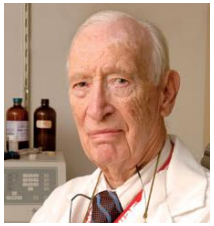
Příslib genových terapií

- Známe generický kód - můžeme léčit jakoukoliv nemoc
- Obecný mechanismus - specifický DNA cíl
- Nejen léčit – vyléčit!
  - Oligonukleotidy (AON, siRNA)
  - mRNA
  - Virové vektory
  - Crisper/Cas 9
  - Modifikované T buňky (CAR-T)

- Individuální terapie
- Vzácné nemoci
- Běžné nemoci

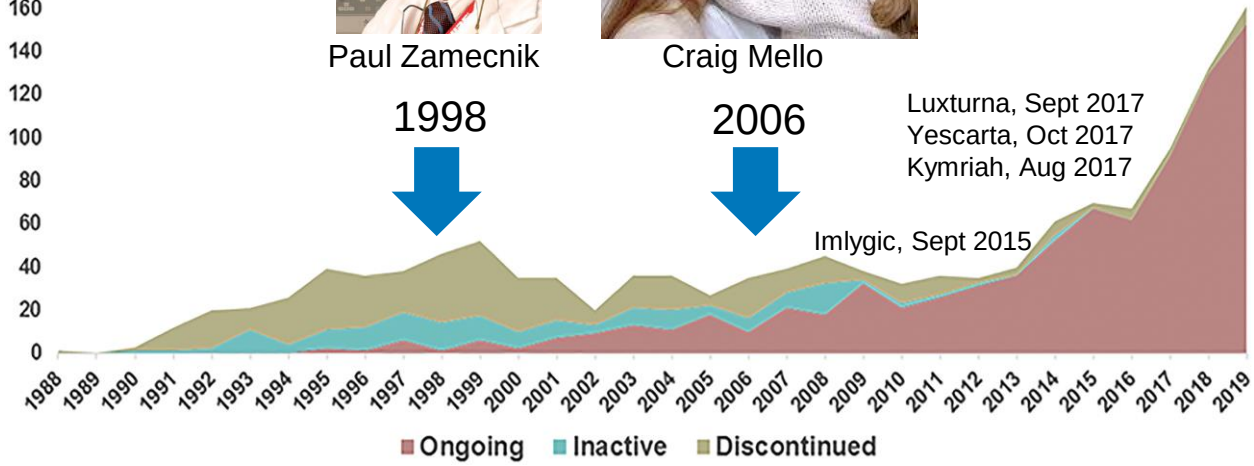


Nové genové terapie



Paul Zamecnik

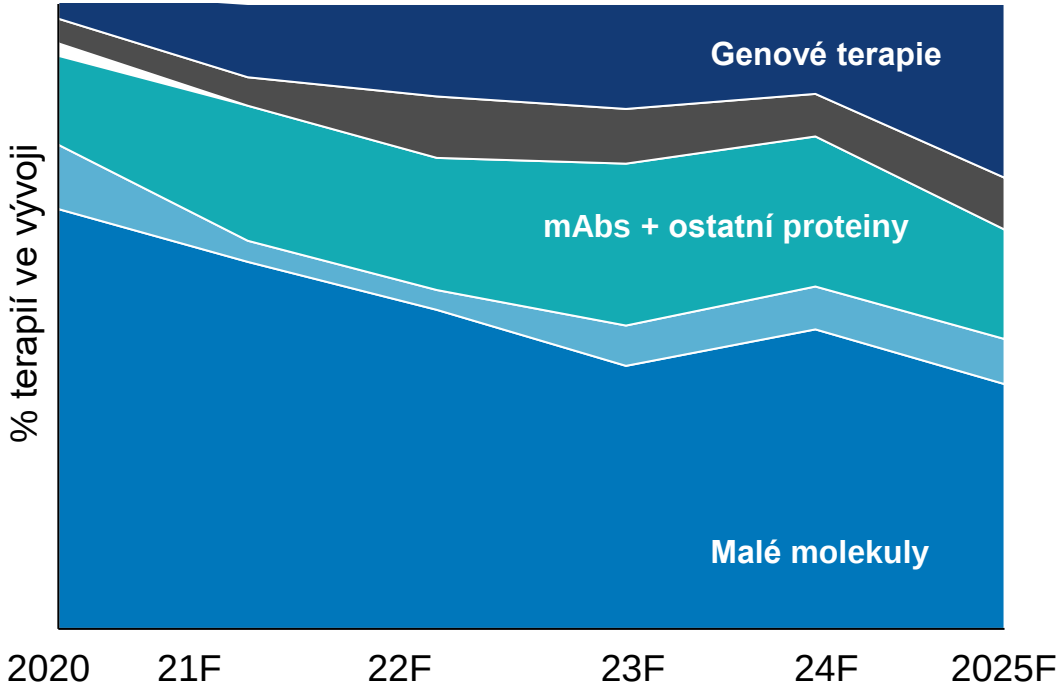
Craig Mello



- **Vyřešené problémy:**
- **Syntéza a biosyntéza**
- **Doprava do buněk a orgánů**
- **Stabilita**
- **Vedlejší účinky a toxicita**
- **Analytické metody**

"Clinical Development of Gene Therapies: The First Three Decades and Counting" Lapteva, et al. 2020.

Nové terapie vstupují na scénu



Genové terapie
Buněčné terapie
mAbs + ostatní proteiny
Jiné
Malé molekuly

Source: Evaluate Pharma, June 2020

Co jsou to Nové Modality?

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Traditional Biotherapeutics

mAb& mAb fragments

150 kD, <150kD

Antibody-Drug Conjugates

~152 kD

Virus Like Particles

No viral genome

Proteins & Peptides

Peptides < 6 kDa

Bispecific & Trispecific mAb, Fusion proteins

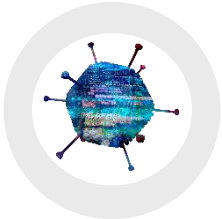
Bi & Tri mAb > 150 kDa

pDNA

607 kDa (1000 bp)

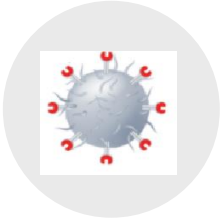
Foundation of biosynthesis; emerging therapeutic/vaccine

Emerging New Modalities
/ Advanced Therapies



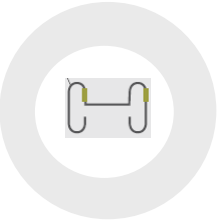
Viral Vectors

Gene Therapy
AAV(ø20nm): 3700 kDa
Adenovirus & Lentivirus (ø90 nm)



Cell Therapy

Most common, CAR-T autologous or allogeneic cells

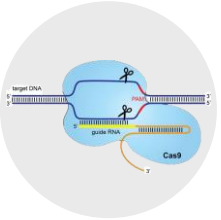


Transgene

Genetic material delivered for CGT

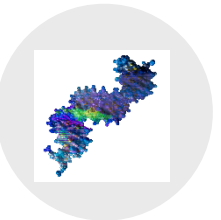
mRNA

960-1600 kDa
(300-5000 nt)
cell-free bioprocess



CRISPR Cas

Gene Editing,
gRNA, 100 nt, 32.2kDa
Cas protein 163 kDa

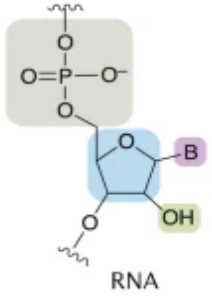


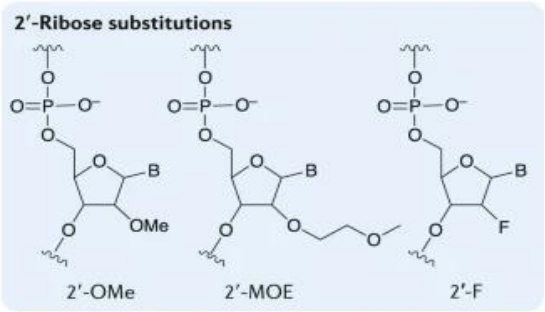
Oligos

Gene Silencing
siRNA, RNAi, ASO
21-23nt; 6.9-7.6kDa

Antisense oligonukleotidy, siRNA, miRNA

Frontiers in Bioengineering and Biotechnology



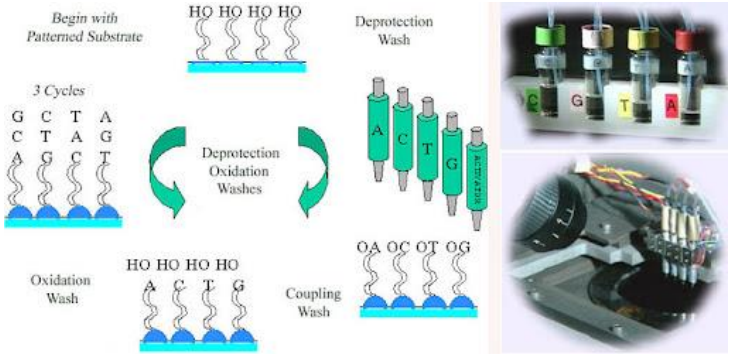


Zdroj: Nature. 2020

www.youtube.com/watch?v=uiObPBeof5s
Richard Jorgensen
Craig Mello, Andrew Fire

Umíme selektivně zapnout a vypnout geny

Syntéza oligonukleotidů – revoluce v biotechnologii



5’ 3’

UUC UGU AAU CUC UUG UCU ATT	(N)
UUC UGU AAU CUC UUG UCU ATT	(N-1)
UUC UGU AAU CUC UUG UCU ATT	(N-2)
UUC UGU AAU CUC UUG UCU ATT	(N-3)
UUC UGU AAU CUC UUG UCU ATT	(N-4)
UUC UGU AAU CUC UUG UCU ATT	(N-5)
UUC UGU AAU CUC UUG UCU ATT	(N-6)

…

Chromatografické metody analýzy oligonukleotidů

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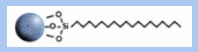
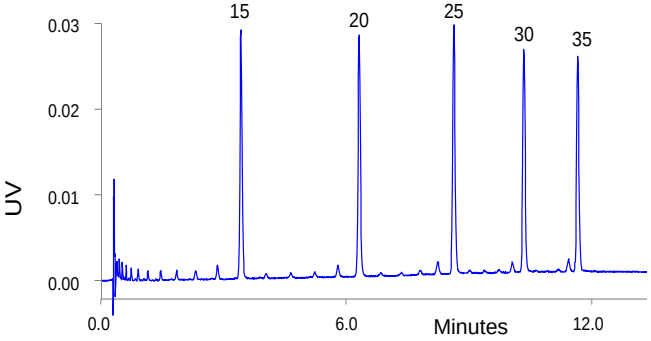
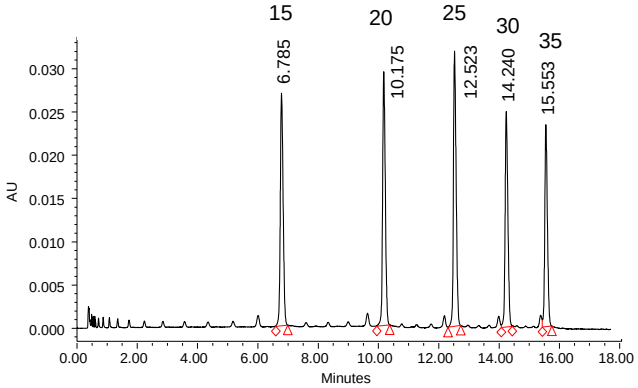


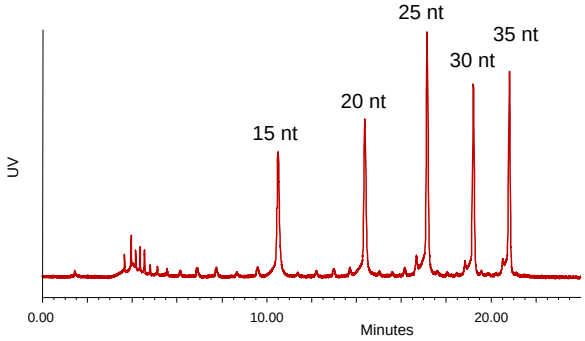
Reversed Phase
Ion Pairing

HILIC

Anion Exchange





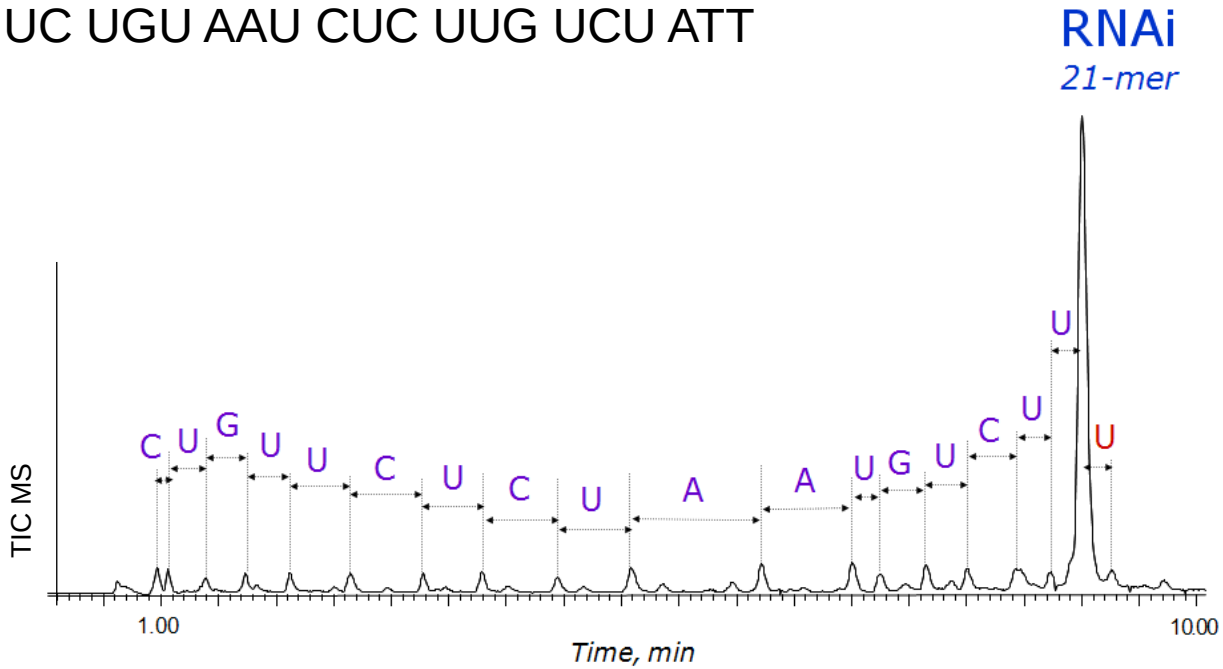


M. Gilar, F. Gritti, J. Nguyen, C. Doneanu, H. Yang, S. Rzewuski, M. Lauber

LC MS aplikace

5’ 3’

UUC UGU AAU CUC UUG UCU ATT



V. Ivleva, Y.Q. Yu, M. Gilar, RCM 2010, 24, 2631

Examples of Application Notes for Oligonucleotides

Additional Application notes in the Waters' "Application Solutions for Oligonucleotides"

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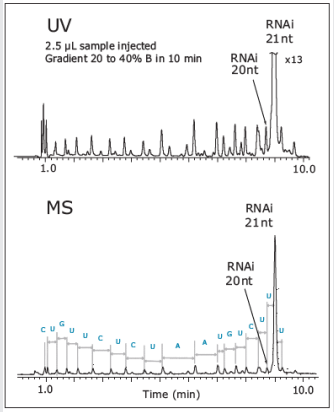
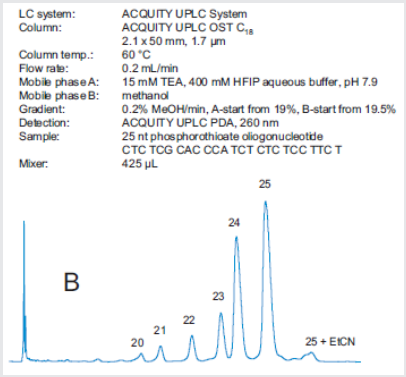
UPLC-MS Analysis of Interfering RNA Oligonucleotides



Detection and quantitation of process-related impurities from synthesis as well as modification or truncations of the oligonucleotide.

https://www.waters.com/webassets/cms/library/docs/720002873en.pdf

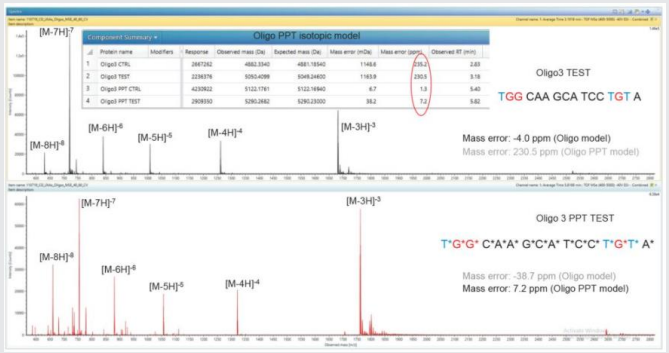
UPLC Analysis of Phosphorothioate Oligonucleotides: Method Development



A single analysis method applied to antisense oligonucleotides that replaces the need for multi-step post purification methods.

https://www.waters.com/webassets/cms/library/docs/720002873en.pdf

Modified Oligonucleotides – intact analysis

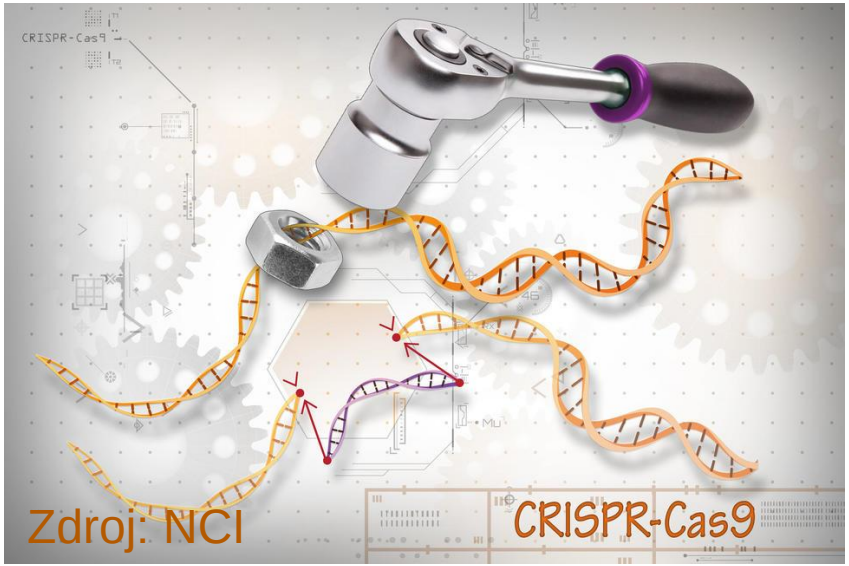


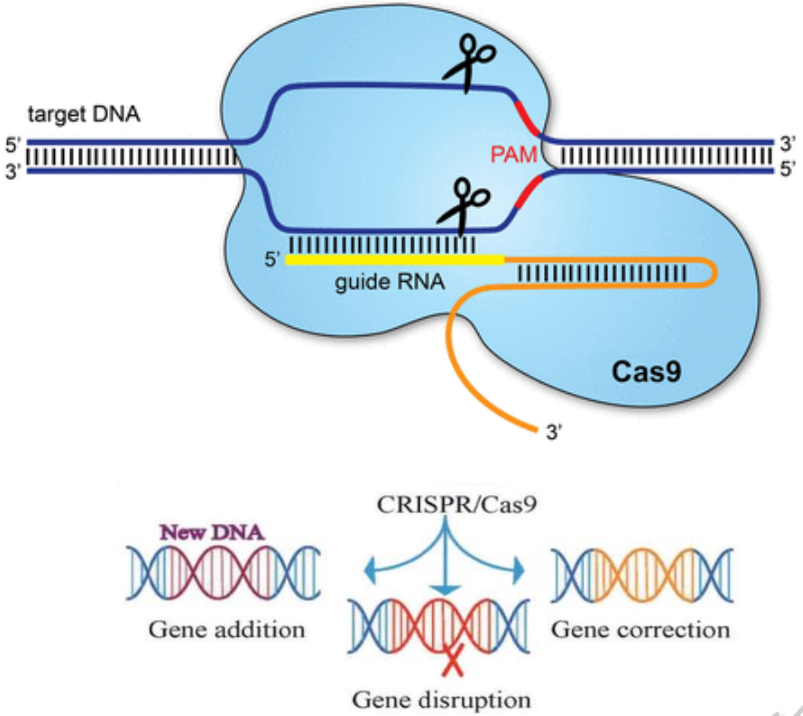
Compliance Ready LC-MS workflow for mass confirmation for unmodified and extensively modified oligonucleotides using the BioAccord.

Intact Mass Confirmation Analysis on the BioAccord LC-MS System for a Variety of Extensively Modified Oligonucleotides

Technologie CRISPR-Cas9 – permanentní editace genů

Clustered Regularly Interspaced Short Palindromic Repeats
Bakteriální obranný mechanismus – „genetická paměť“
Cas9 – nukleáza, štípe dsDNA
100 mer guideRNA (gRNA) – určí štěpné místo
Jennifer Doudna 2020 Nobel prize





Separace 5-100 dT oligonukleotidů

ACQUITY PREMIER Oligonucleotide BEH C18, 130 Å, 1.7 µm, 2.1 x 150 mm column

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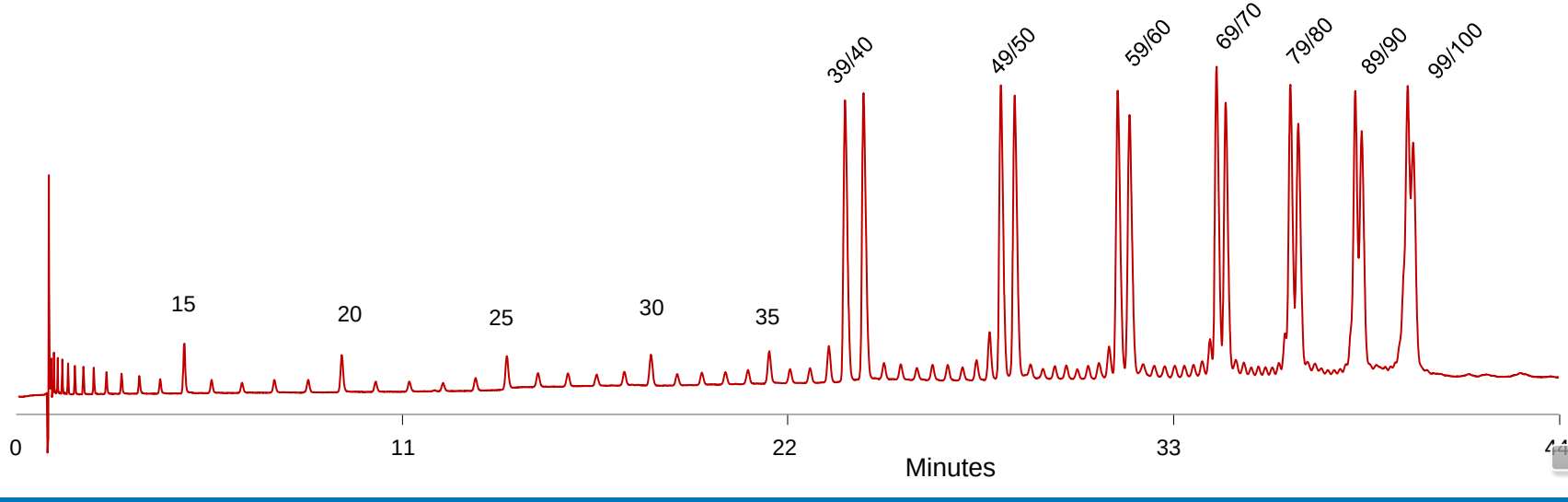
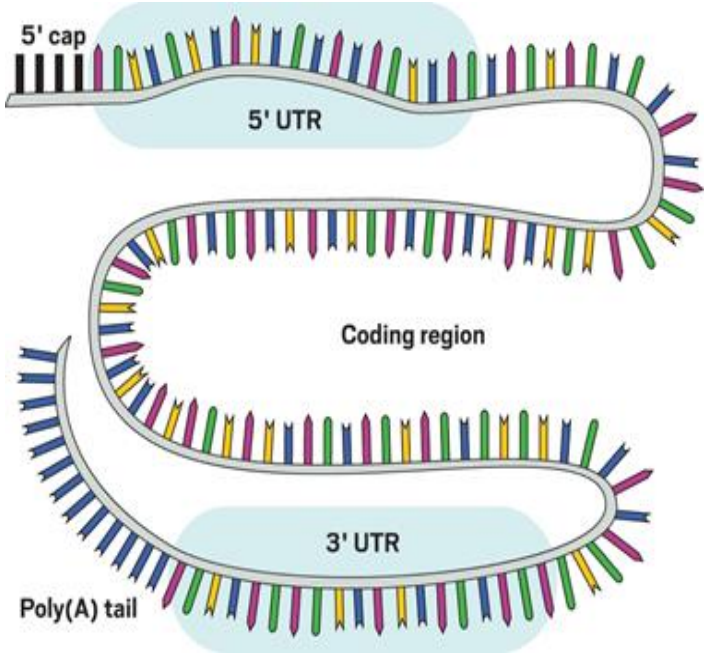
Mobile phase A: 100 mM Hexylammonium acetate pH 8 (HAA) : MeCN, 75 : 25 (v:v)
Mobile phase B: 100 mM HAA : MeCN, 25 : 75 (v:v)
Conditions: 60 °C, 0.4 mL/min, UV 260 nm, gradient 15-50% B in 60 minutes



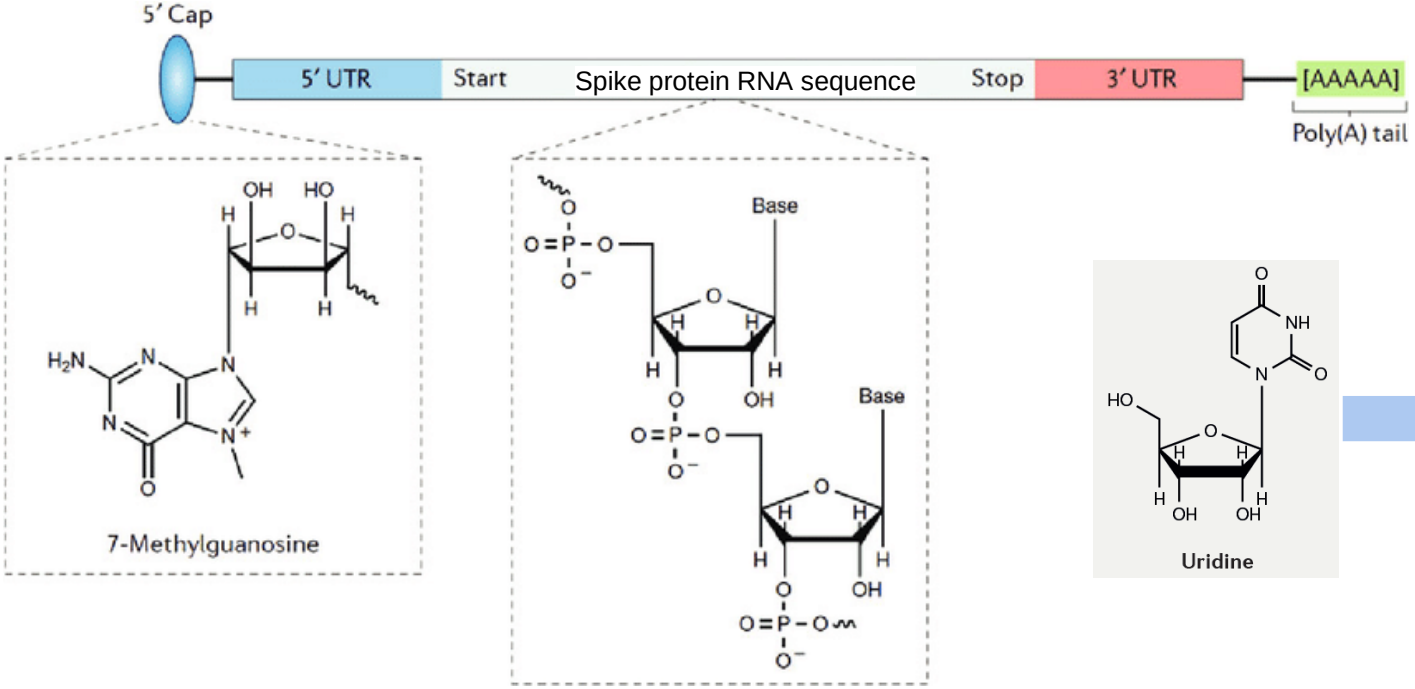
Anatomie mRNA - principy mRNA technologie



- 1970’ – struktura a funkce 3’ polyA konce
- 1970’ – struktura a funkce 5’ “čepice”
- 1985 – biosyntéza mRNA (T7-RNA polymeráza)
- 1987 – komercializace PCR a Taq polymerázy
- 1980-2010’ – vývoj lipidových nanočástic
- 2005 – modifikovaná mRNA - minimálně imunogenní
- 2014 – mRNA technologie funguje

Katalin Karikó (BioNtech)

Struktura Covid 19 mRNA – mRNA syntetizuje Spike protein



LC-UV Chromatograms of mRNA using IP-RPLC

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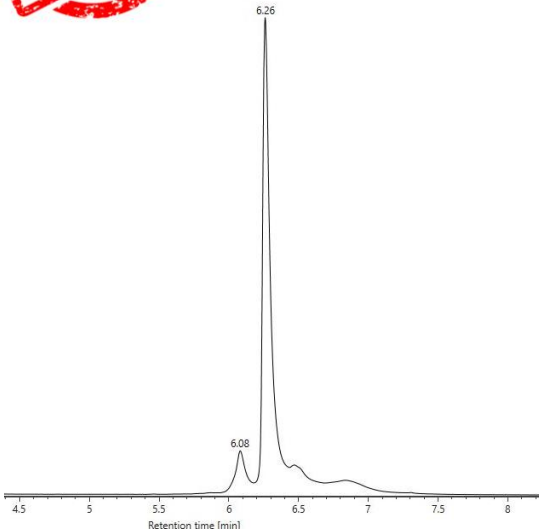
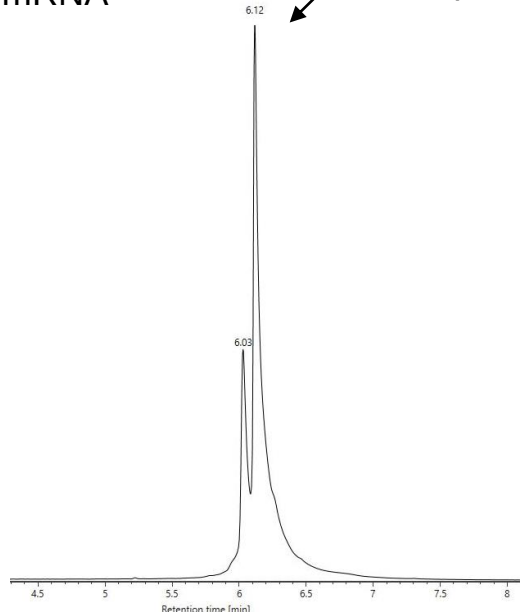
ACQUITY Premier Oligonucleotide Column, 2.1 x 50 mm, 1.7 µm, 130 Å, DIPEA/HFIP Mobile Phase System

PATENT PENDING

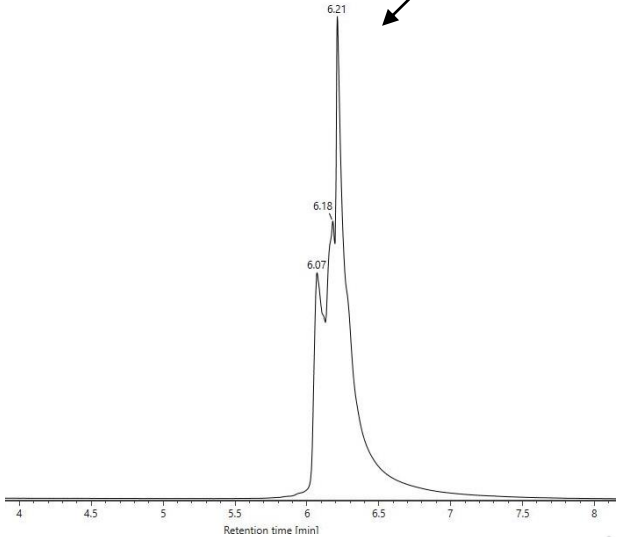
TriLink EPO (human erythropoietin) mRNA (858 nt)



TriLink Luc (firefly luciferase) mRNA (1929 nt)



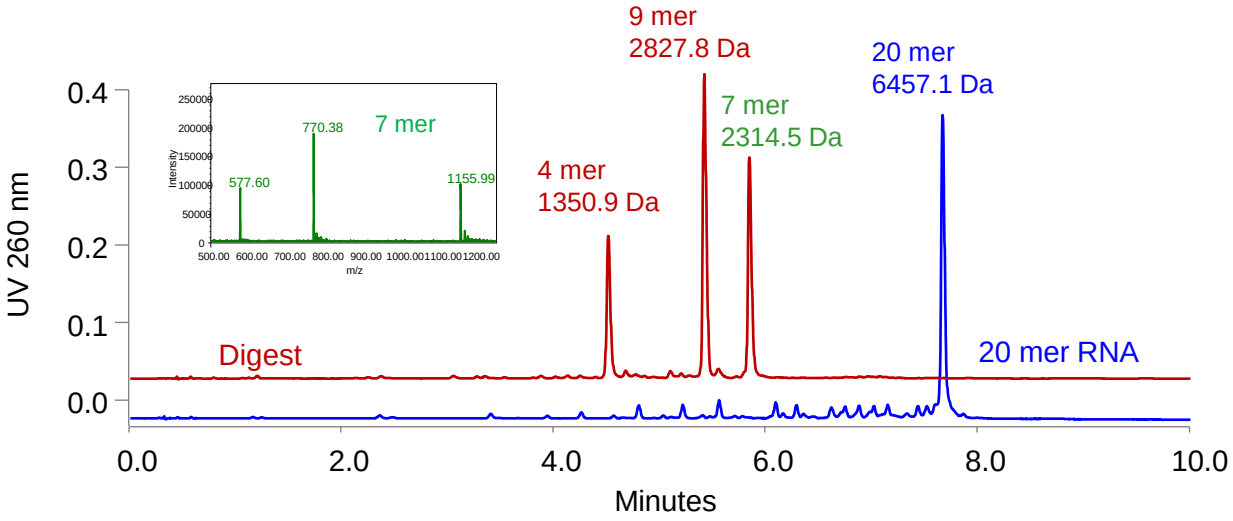
TriLink Cas-9 (CRISPR associated protein 9) mRNA (~ 4,521 nt)



Data: Catalin Doneanu

HILIC analysis of RNase T1 digest

rArArArGrArArArArCrArGrArArArUrArArATT	20 mer, 6457.1 Da
rArArArG–PO_3H	4 mer, 1350.9 Da
rArArArArCrArG–PO_3H	7 mer, 2314.5 Da
ArArArUrArArATT	9 mer, 2827.8 Da



EPO mRNA mapping – RNase T1 digest

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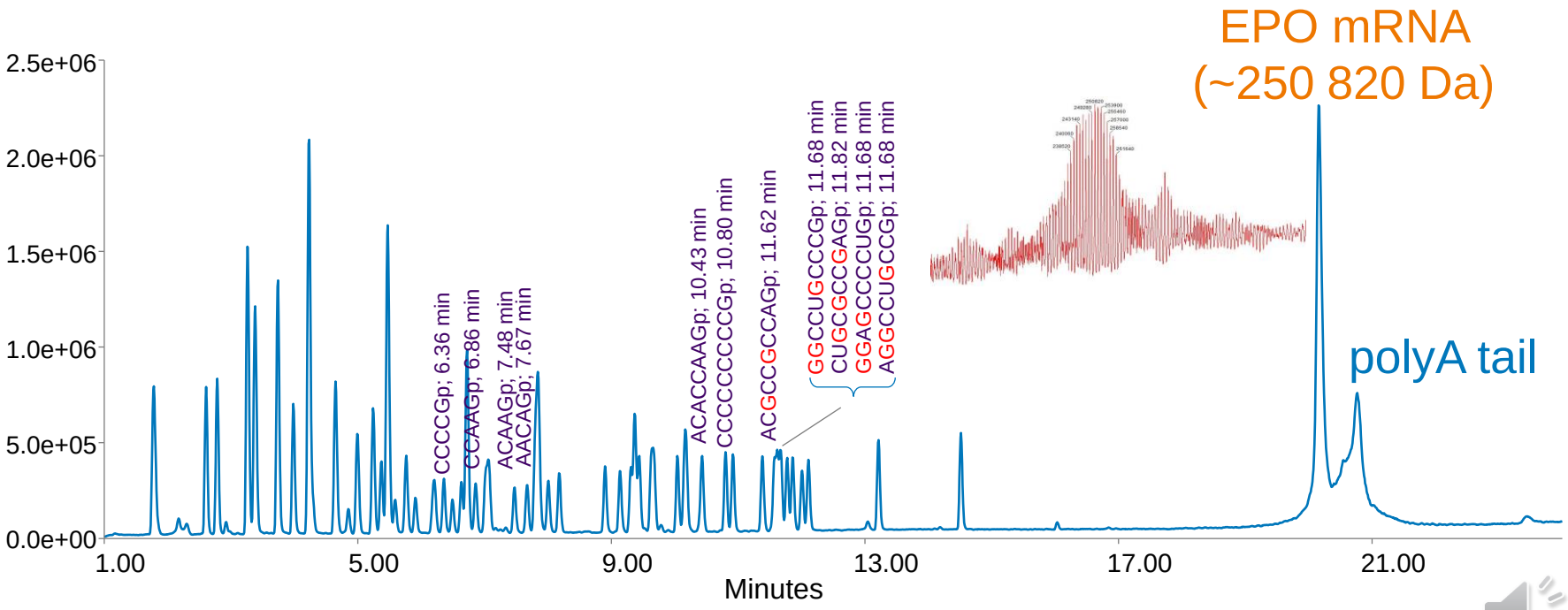
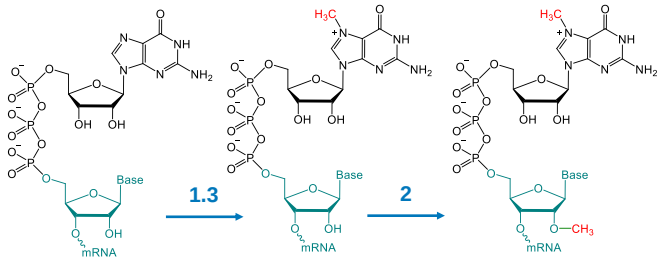
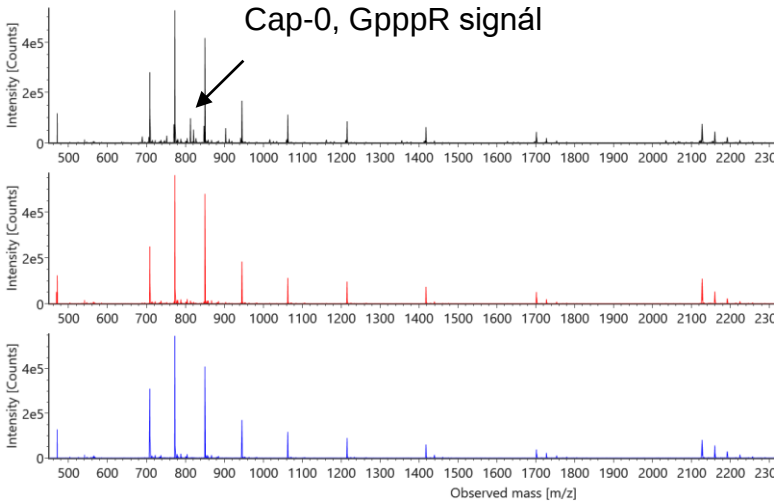


LC MS data: Catalin Doneanu

RNase H digest, 5’Cap LC-MS analýza

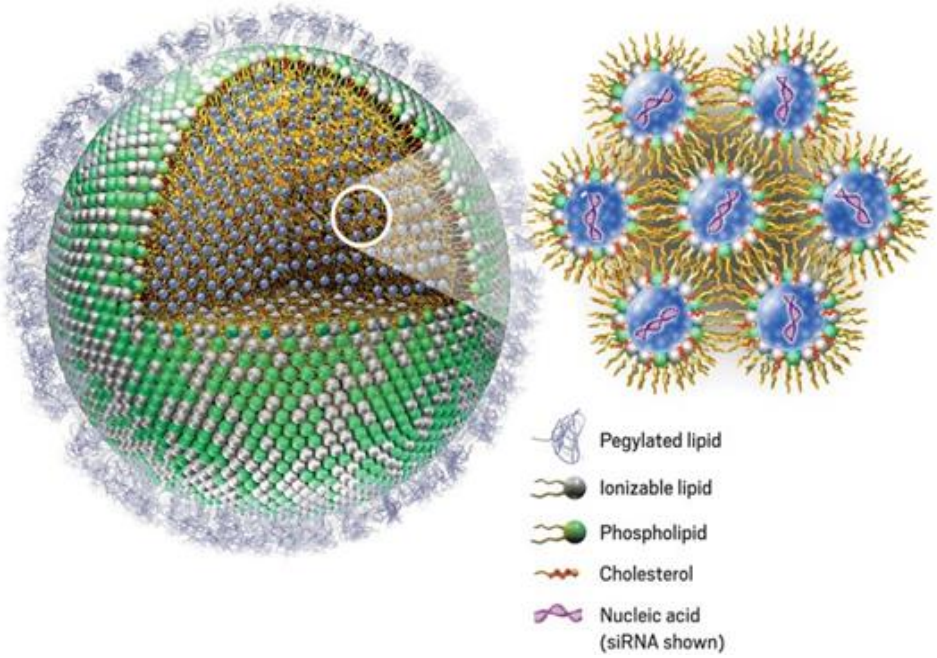


Base = guanine or adenine
R = guanosine or adenosine



LC MS data: Jennifer Nguyen

Doprava mRNA do buněk



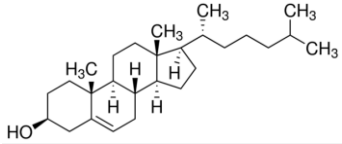
50%

Cationic Lipid (Patented)

MW 641.6111 Da

38%

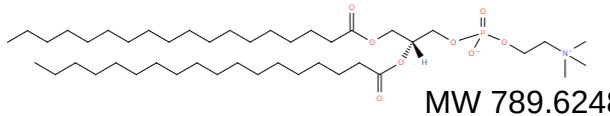
Cholesterol



MW 386.3549 Da

10%

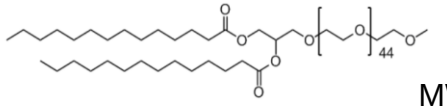
Phospholipid (18:0/18:0) PC



MW 789.6248 Da

2%

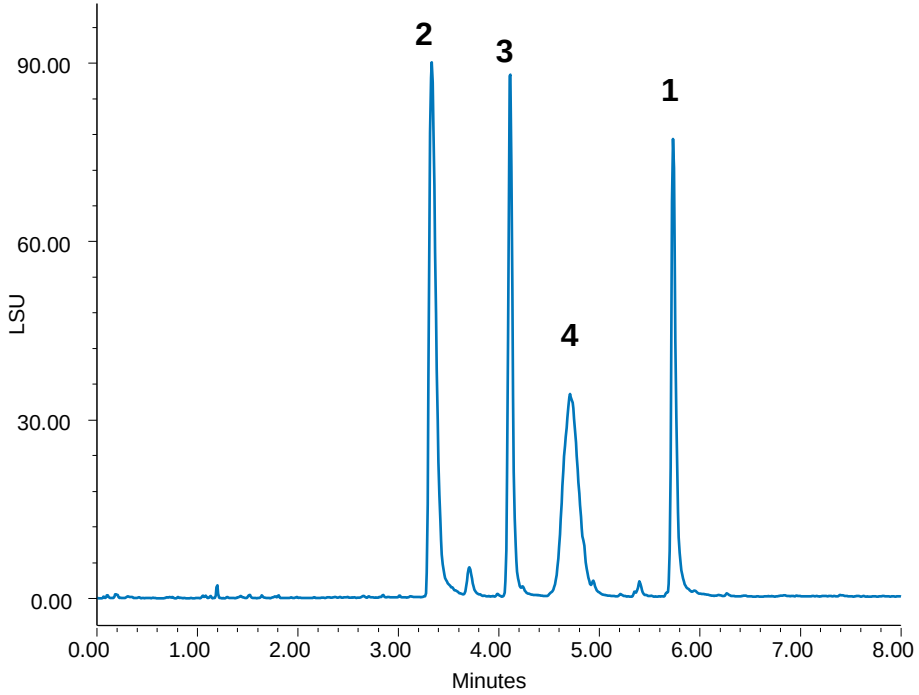
PEGylated Lipid



MW 2507.640 Da

Složení lipidových nanočástic - LC ELSD

2.1 x 50 mm,1.7 µm CSH Phenyl-Hexyl kolona



[1] 1,2-distearoyl-sn-glycero-3-phosphocholine
[2] (6Z,9Z,28Z,31Z)-heptatriacont-6,9,28,31-tetraene-19-yl 4-(dimethylamino)butanoate
[3] Cholesterol USP
[4] 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000

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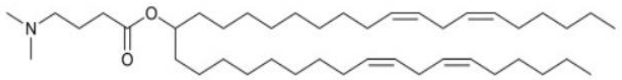
Cationic Lipid (Patented)

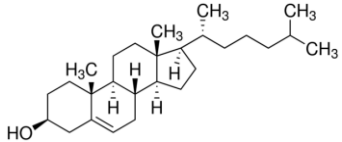
2



MW 641.6111 Da

Cholesterol

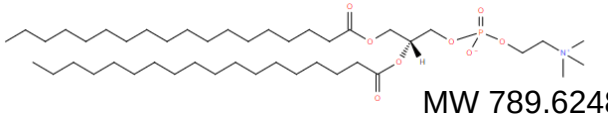
3



MW 386.3549 Da

Phospholipid (18:0/18:0) PC

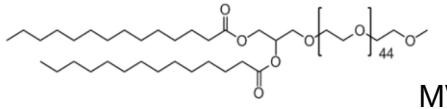
1



MW 789.6248 Da

PEGylated Lipid

4



MW 2507.640 Da

mRNA vakcíny: Úspěch otevírá dveře dalším terapiím

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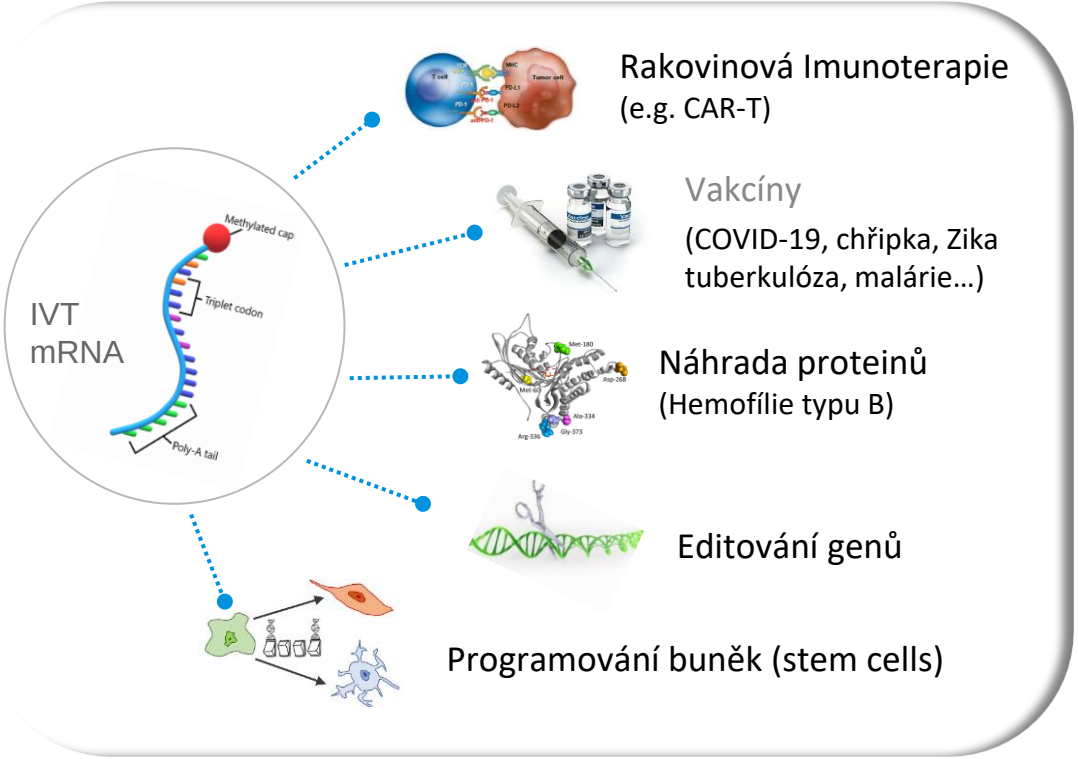


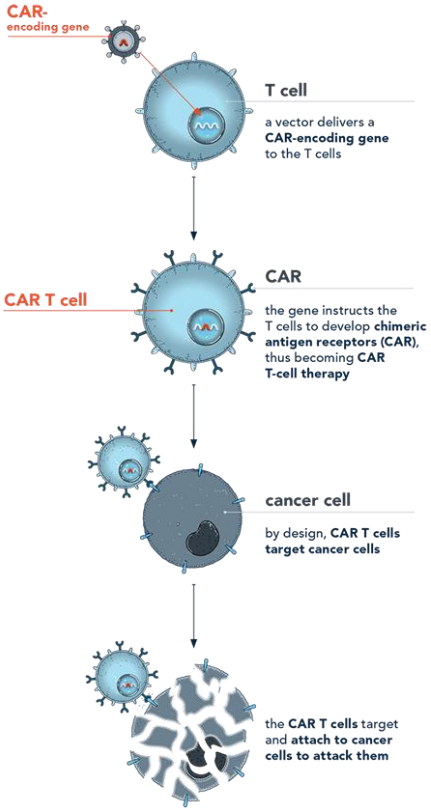
Rakovinová Imunoterapie
(e.g. CAR-T)

Vakcíny
(COVID-19, chřipka, Zika
tuberkulóza, malárie…)

Náhrada proteinů
(Hemofílie typu B)

Editování genů

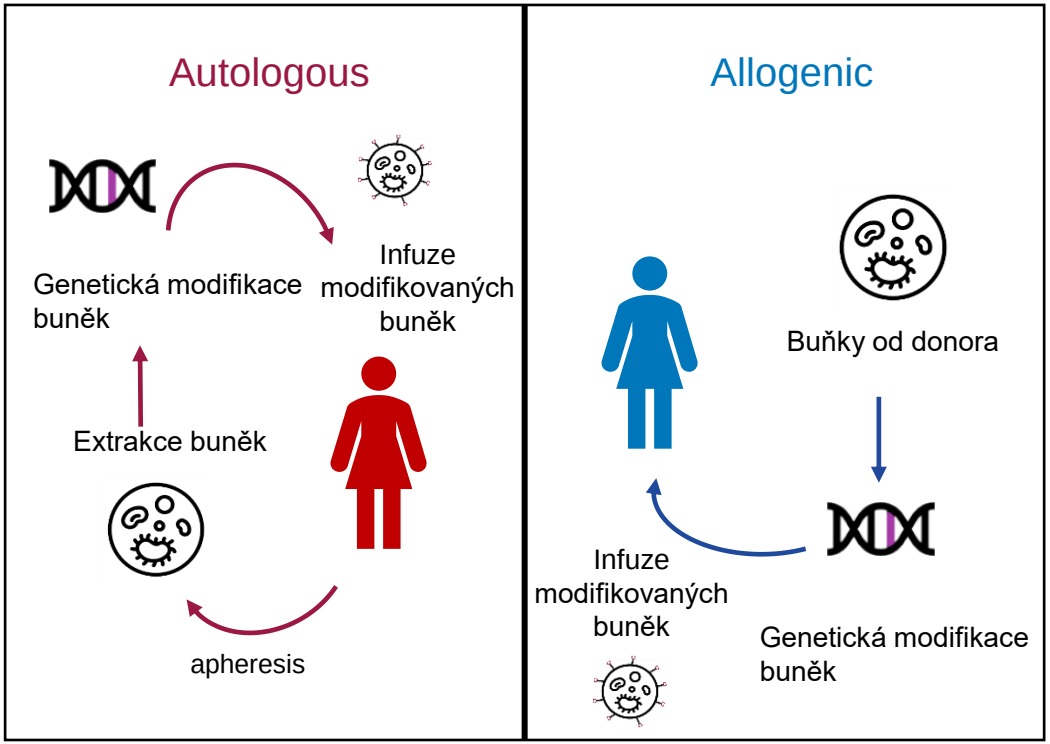
Programování buněk (stem cells)



Zdroj: Hajj & Whitehead NATURE REVIEWS | MATERIALS 2017

Kymriah: Treatment for B-cell acute lymphoblastic leukemia
Yescarta: Treatment for B-cell lymphoma

CAR-T: Chimeric antigen receptor T-cell



- Ex-vivo modifikace
- Pouze T-buňky (T-lymfocyty) jsou modifikovány; mRNA vyprodukuje CAR protein
- mRNA je meziprodukt pro ssDNA kódující Crispr-Cas9 komplex. ssDNA je vložena do T-lymfocytů. Crispr-Cas9 přeprogramuje buňku na CAR T-cell
- Ex-vivo postup minimalizuje možnost nežádoucí genové změny „mimo cíl“ - v jiných typech buněk
- 5 komerčních Car-T terapií

Anatomie virových vektorů

DNA je vložena do buněčného jádra

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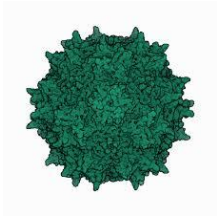
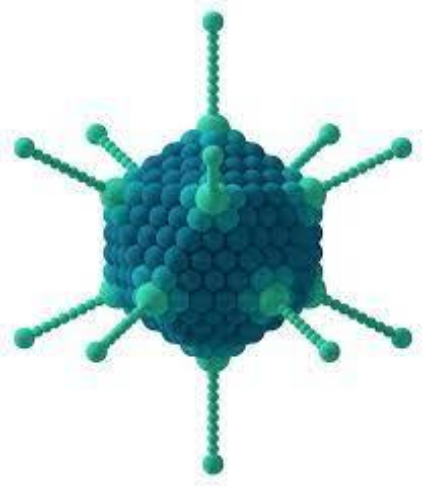
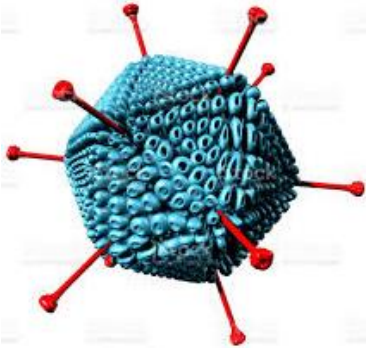




- Adeno Associated Viry (AAV)
- Malé nepatogenní viry, hmotnost 4.8 MDa (ekvivalent 32 mAb)
- AAV se skládá z 60 proteinů
- VP1:VP2:VP3 ~ 5:5:50
- VP MW: 60, 67, 82 kDa; 25 nm, velikost 2.5x mAb
- Nálož ssDNA, 4.5 kb limit

- Adeno Viry (AdV)
- Středně velké viry, hmotnost 150 MDa (ekvivalent 100 mAb)
- AdV se skládá z 252 proteinů, 240 hexonů, 12 pentonů
- 129 kDa, 51.1 kDa; velikost 90-100 nm, 4x mAb
- Nálož dsDNA, 32-43 kb
- Sputnik V (AdV26, AdV5), Janssen (AdV26), AstraZeneca (AdV26)

Analýza virových částic

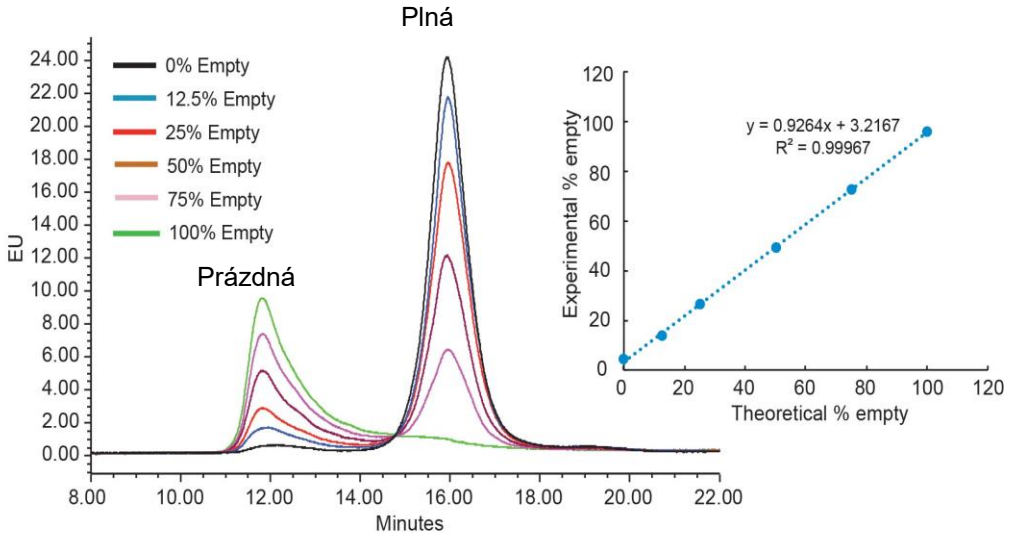


Zdroj: istockphoto.com

Prázdná
AAv obálka

Nekompletní
DNA obsah

AAv s
cílovou DNA



Hua Yang et al., Waters 720006825

LC and LC/MS technologie pro kontrolu AAV terapií

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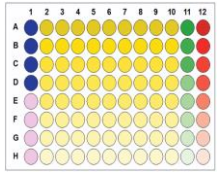
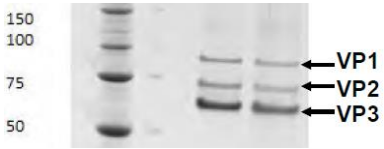
ELISA
Anti-Capsid Antibody Binding

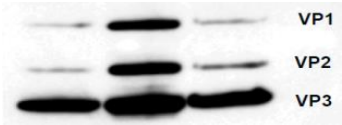


AUC
Measure difference in Sedimentation Coefficient

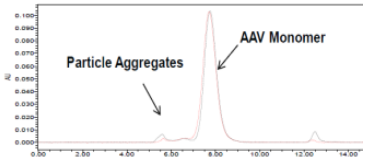
SDS-PAGE
Measure difference in separation profile



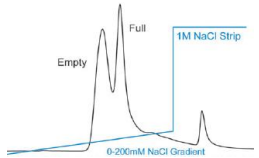
Western Blot
Resource intensive



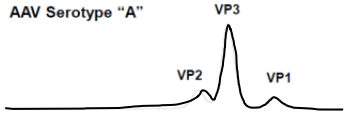
SEC-FLR-UV
Purify and measure intrinsic fluorescence



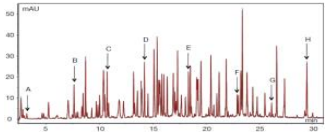
IEX-UV
Measure difference in surface charge profile



RPLC-UV-MS
Measure difference in hydrophobicity profile



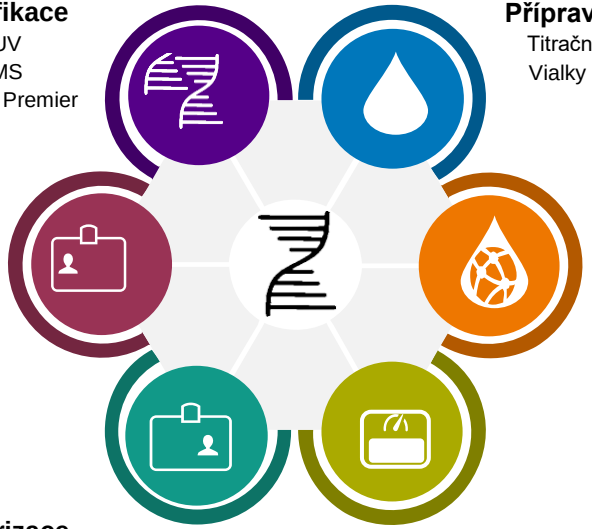
Peptide Mapping
AA sequence and PTMs



Analýza oligonukleotidů a nukleových kyselin

Kvantifikace
LC UV
LC MS
ACQUITY Premier

Příprava vzorků
Titrační destičky
Vialky MaxPeak



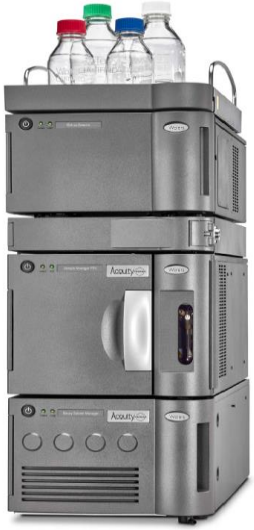
Separace
Iontově-párové systémy
OST vzorky

Kolony Premier
BEH C18 OST
BEH Phenyl
BEH AMIDE HILIC

Charakterizace
Sekvencování/ analýza modifikací
LC/MS ToF, QTof

Speciální aplikace
SEC
IEX
RP analýza nukleotidů

Acquity PREMIER



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Analýza nukleových kyselin s HPS technologií

High Performance Surfaces (HPS)

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analytical chemistry

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Using Hybrid Organic–Inorganic Surface Technology to Mitigate Analyte Interactions with Metal Surfaces in UHPLC

Mathew DeLano, Thomas H. Walter,* Matthew A. Lauber, Martin Gilar, Moon Chul Jung, Jennifer M. Nguyen, Cheryl Boissel, Amit V. Patel, Andrew Bates-Harrison, and Kevin D. Wyndham

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CHROMATOGRAPHY TODAY February / March 2021

Faster Time to Results for Ultra-Performance Liquid Chromatographic Separations of Metal-Sensitive Analytes

Thomas H. Walter, Brian J. Murphy, Moon Chul Jung†, Martin Gilar, Robert E. Birdsall and Jacob Kellett, Waters Corporation 34 Maple Street, Milford, MA 01757

† Current address: Enanta Pharmaceuticals Inc, 500 Arsenal Street, Watertown, MA

ELSEVIER Journal of Chromatography A 1650 (2021) 462247

Mitigation of analyte loss on metal surfaces in liquid chromatography

Martin Gilar*, Mathew DeLano, Fabrice Gritti

Waters Corporation, 34 Maple Street, Milford, MA 01757, USA

Assessing the impact of nonspecific binding on oligonucleotide bioanalysis

Bioanalysis

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Bioanalysis (2021) 13(16), 1233–1244

Journal of Pharmaceutical and Biomedical Analysis

208 (2021) 114439

Impact of Nonspecific Adsorption to Metal Surfaces in Ion Pair-RP LC-MS Impurity Analysis of Oligonucleotides

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