SLOVENSKO FARMACEVTSKO DRUŠTVO
SEKCIJA FARMACEVTSKIH TEHNOLOGOV

Univerza v Ljubljani
Fakulteta za farmacijo

29. simpozij Sekcije farmacevtskih tehnologov
29th Symposium of Pharmaceutical Technology Section

and

CEEPUS Summer School, Ljubljana, June 2018
Central European Knowledge Alliance for
Teaching, Learning & Research in Pharmaceutical Technology

Polimeri kot pomožne snovi in učinkovine

Polymers as Pharmaceutical Excipients and Active Ingredients

ZBORNIK PREDAVANJ
PROCEEDINGS

14. 6. 2018, Ljubljana

Sponzorji 29. simpozija Sekcije farmacevtskih tehnologov

Sponsors of the 29th Symposium of the Pharmaceutical Technology Section

Sponzorjem se iskreno zahvaljujemo za njihovo tradicionalno podporo!
Sponsors support is highly appreciated !

29. simpozij Sekcije farmacevtskih tehnologov z naslovom Polimeri kot pomožne snovi in učinkovine združuje področji, ki obravnavata polimerne materiale po njihovi različni primarni vlogi v farmacevtskem izdelku. Na podlagi pozitivne izkušnje iz lanskega simpozija, ki je obsegal dve temi, si obetamo, da bo široko področje obravnave ponovno privabilo veliko število udeležencev.

V prvem delu simpozija se bomo usmerili v področje polimernih ekscipientov. Beseda bo tekla o njihovih lastnostih, ki lahko ključno vplivajo na proces izdelave končnega izdelka ali na njegove končne lastnosti. Spoznali bomo nekatere analizne metode za proučevanje polimernih pomožnih snovi, novosti s področja oblikovanja hipromeloze acetat sukcinata, s predavanjem o nanocelulozi pa se bomo dotaknili prihodnosti ter preverili, kakšne možnosti nam tak material nudi za uveljavljanje novih pristopov pri oblikovanju zdravil.

Drugi del simpozija je namenjen polimerom, ki jih v formulacije vključujemo kot zdravilne učinkovine. Strokovnjaki s področja industrije in akademskih krogov nam bodo predstavili fizikalne, kemijske in biološke lastnosti proteinskih učinkovin, tvorbo kompleksov in možnost modifikacije za izboljšano delovanje ter možnosti proizvodnje bioloških zdravilnih učinkovin. Pogledali bomo tudi v področje razvoja proteinskih kompleksov nanometrskih velikosti in njihovo uporabo v biotehnologiji.

Člani strokovno - organizacijskega odbora:
dr. Natalija Škrbina Zajc
prof. dr. Stane Srčič
dr. Zrinka Abramović

PROGRAM

8.00 – 8.45	Registracija / Registration
9.00	dr. Natalija Škrbina Zajc, predsednica strokovno - organizacijskega odbora Uvodni pozdrav / Intoduction

Polimeri kot pomožne snovi / Polymers as Pharmaceutical Excipients

9.10 – 9.45	dr. Sabina Devjak Novak **S funkcionalnostjo povezane lastnosti polimerov kot pomožnih snovi** **Functionality related characteristics (FRC) of polymers as pharmaceutical excipients** *Krka, tovarna zdravil, d.d., Novo mesto*
9.50 - 10.25	dr. Jörg Brunemann **Aqoat (HPMC-AS) / Trdne disperzije / Sušenje z razprševanjem / Iztiskanje talin** **Aqoat (HPMC-AS) / Solid dispersions / Spray drying / Hot melt extrusion** *Harke Pharma GmbH, Müllheim, Nemčija*
10.30 – 11.00	Odmor / Coffee Break
11.00 – 11.35	dr. Matjaž Kunaver **Nanoceluloza – biomaterial bodočnosti** **Nanocellulose – biomaterial of the future** *Kemijski inštitut, Ljubljana*
11.40-12.15	dr. Boštjan Jerman **Karakterizacija polimerov – analitski pristopi v farmacevtski industriji** **Polymer characterization - analytical approach in pharmaceutical industry** *Krka, tovarna zdravil, d.d., Novo mesto*

Polimeri kot zdravilne učinkovine / Polymers as Active Ingredients

12.20 – 12.50	doc. dr. Tomaž Bratkovič **Fizikalno-kemijske, strukturne in biološke lastnosti proteinskih učinkovin** **Physicochemical, structural and biological properties of proteins as active ingredients** *Univerza v Ljubljani, Fakulteta za farmacijo*
12.55 - 14.00	Kosilo / Lunch
14.00 – 14.35	dr. Barbara Podobnik **Razvoj imunokonjugatov za ciljano terapijo raka** **The development of immunoconjugates for targeted cancer therapy** *Lek farmacevtska družba d.d., Ljubljana*
14.40 – 15.15	doc. dr. Marjetka Podobnik **Proteinski kompleksi nanometrskih velikosti in njihova uporaba v biotehnologiji** **Nanosized protein complexes in biology and their application in biotechnology** *Kemijski inštitut, Ljubljana*
15.20 – 15.55	dr. Simona Jevševar **Izboljšava terapevtskega delovanja proteinov s proteinsko modifikacijo** **Improvement of therapeutic proteins by protein modification** *Lek farmacevtska družba d.d., Ljubljana*
16.00	Sklep simpozija / End of Symposium and Conclusions

KRKA

Živeti zdravo življenje.

S funkcionalnostjo povezane lastnosti polimerov kot pomožnih snovi

Functionality related characteristics (FRC) of polymers as pharmaceutical excipients

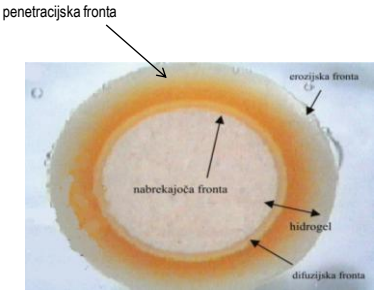
dr. Sabina Devjak Novak

Simpozij sekcije slovenskih tehnologov 2018

14. junij 2018

EXCIPIENTS

- Definition: substances with significant contribution to physico-chemical properties of pharmaceutical formulation, desirable kinetics and extent of absorption of API
- Swelling of polymer, controlled release of API:



-after getting in contact with water, polymer transforms from glassy state into soften state (Tg)
-elastic hydrogel formation
-highly increased mobility of polymer chains
-formation of different contact surfaces inside elastic hydrogel
-release of API with diffusion, erosion or combination of both mechanisms

EXCIPIENTS

- Enable easier application of medicine
- Inert, stable, physiologically acceptable
- Necessary in every pharmaceutical dosage form (solid, semisolid, liquid)
- Representing from 1% to 99% of the total weight
- Use:

  -to improve manufacturing procedure,

  -to optimize the appearance and taste of final formulation,

  -to ascertain suitable stability of the product

  -to achieve target characteristics of formulation,

  -to increase patient compliance

Functionality related characteristics (FRC) of excipients

- Chapter included in European Pharmacopoeia Ed. 6th – not mandatory, published for information
- Chapter included in Formularium Slovenicum
- General monography **5.15 Functionality-related characteristics of excipients** (FRC) with following sections:

  -Preamble

  -Regulatory guidance

  -Physical grades

  -Chemical grades

  -FRC section in monographs

  -International harmonization

  -Glossary

Functionality related characteristics (FRC) of excipients

- Guideline ICH Q8 – influence on Pharmaceutical Development:

  quality by testing ➡ quality by design

- Changes in monographies
- European Pharmacopoeia: functionality related characteristics, individual pharmaceutical formulation/manufacturing procedure ➡ numerous analytical techniques
- Expression „*Functionality related characteristics*“ or „*FRC characteristics*“: ➡ ***physical and chemical characteristics of excipient, related to functionality; can be controlled – a part of product specification***
- Function of excipient in formulation and during the manufacturing process
- Manufacturer‘s duty to define, which FRC data and how will be used during the development of new product, also considering the technological procedure

Studies of FRC in excipients

- Comparison of physicochemical characteristics of hydroxypropyl cellulose (HPC) samples of one or more manufacturers
- Significant differences in rheological characteristics of Carbopol 934, but no differences in IR-patterns, density and carboxylic acid content
- Batch-to–batch variations in physicochemical characteristics of microcrystalline cellulose, spray-dried lactose and magnesium stearate of the same manufacturer using PCA analysis (material suitably corresponded to specification demands)

Functionality related characteristics of Hypromellose

- Changes in monography
- Hypromellose as a hydrophilic gel-former → FRC: viscosity, molecular mass distribution, particle size, powder flowability and degree of substitution.
- The most studied characteristics:

Viscosity

Particle size distribution

Degree of substitution

Just additional testing?

Hypromellose, hydroxypropyl methylcelluloze, HPMC

- Overview of hypromellose types according to Ph.Eur. and USP:

methyl groups — hydroxypropyl groups

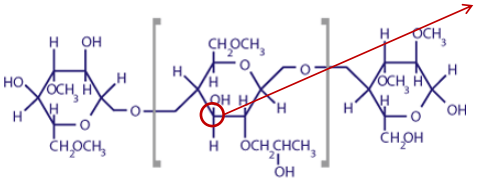
HPMC: Colorcon/Shin-Etsu	Type sub.	Methyl (%)	Hydroxypropyl (%)
Methocel K/ Metolose 90SH	2208	19.0-24.0	4.0-12.0
Methocel E/ Metolose 60SH	2910	28.0-30.0	7.0-12.0
Methocel F/ Metolose 65SH	2906	27.0-30.0	4.0-7.5

HPMC: Colorcon/Shin-Etsu	Viscosity (mPas)
Methocel K4M/ Metolose 90SH-4000	3000-5600
Methocel E4M/ Metolose 60SH-4000	3000-5600
Methocel F4M/ Metolose 65SH-4000	3000-5600
Methocel K15M/Metolose 90SH-15000	12000-21000
Methocel K100M/Metolose 90SH-100000	80000-120000

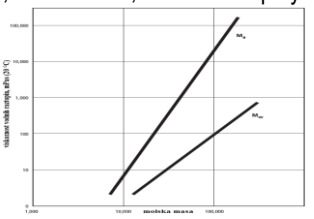
- broad specification limits
- viscosity of 2% w/w colloidal dispersions

Functionality related characteristics of Hypromellose

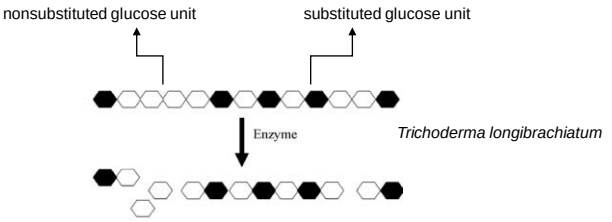
- Structural formula:



- semisynthetic, linear, non-ionic, water soluble hydrophilic cellulose ether, applicable more than 60 years; hygroscopic, inert, non-toxic, non-irritable, viscoelastic polymer
- Cellulose monomer-units connected with 1,4-β glycosidic bond
- Viscosity – the most studied characteristic : influence of molecular mass on viscosity of water dispersions of hypromellose



Functionality related characteristics of Hypromellose – literature data

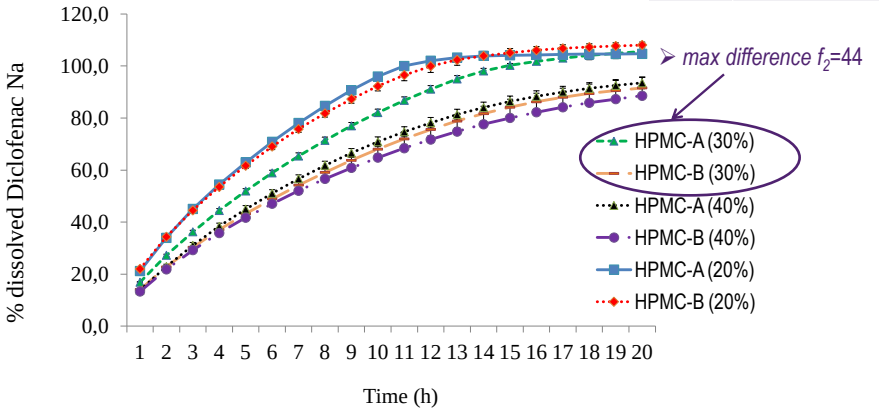


- Statistically different distribution of substituents along polymer chains of hypromellose, more heterogenic substituted patterns erode more slowly, numerous nonsubstituted areas cause different solubility along polymer chains. New FRC: *distribution of substituents along polymer chain* (A. Viriden)
- Influence of particles size and shape at three different types of Hypromellose (2208, 2910, 2906 – different degree of substitution): type 2910 with biggest share of spherical particles; different amount of hydrophobic methoxy groups in particles with different shape (spherical vs. needle-like) of the same hypromellose type (C. Caramella)
- Morphology: influence on mechanical characteristics of matrix tablets; needle-like particles form stronger matrix with slower API release (K. Mitchell)

VISCOSITY studies as FRC parameter…1

➢ Dissolution profiles, Hypromellose type 2208, 4000 mPas

A	2412±60 mPas
B	4464±62 mPas

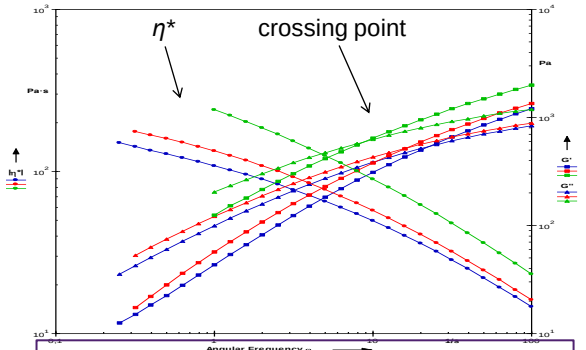


 The influence of HPMC viscosity as FRC parameter on the release of low soluble drug from hydrophylic matrix tablets; PDT 2013; 18(2):343-347

VISCOSITY studies as FRC parameter…2

➢ Frequency tests of 4% in 6% hypromellose water dispersions (2208, 2910 in 2906; 4000 mPas)

➢ pri γ=0.3%, sinusno spr. frekvenci v=0.628-628 Hz, T=20°C±0.5°C



6% w/w dispersions of samples:
2208*, *2910* and *2906

➢ fingerprint of material

➢ inner structure of polymer disperions

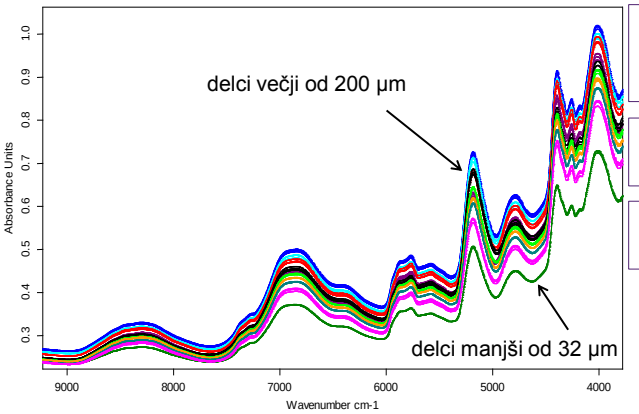
➢ G` in G`` very frequency dependent

➢ Crossing point-new FRC parameter

 Investigation of functionality related characteristics of Hydoxypropyl Methylcellulose of different substitution types; JPBA 2012; 66: 136-143

Results… considering 3rd Hypothesis

- NIR spectra of different hypromellose particle size fractions



spectra in the range: 12500 do 3500 cm^{-1}

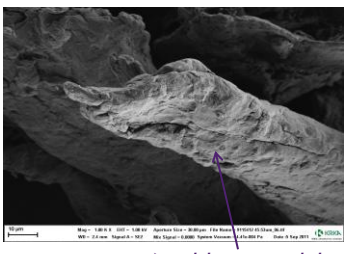
all spectra have the same shape

bigger particles - higher absorption

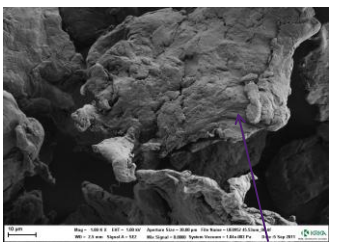
Characterization of physicochemical properties of HPMC type 2208 and their influence on the prolonged drug release from matrix tablets; JPBA 2012 (66): 136-143

PARTICLE SIZE studies as FRC parameter in Hypromellose

- 2208, 4000 mPas; two manufacturers
- shape, particle size distrubution and degree of substitution of Hypromellose različnih velikostnih frakcij
- Scanning electron mycroscopy: images of fractions 32 – 45 µm



➢ *oblong particles*



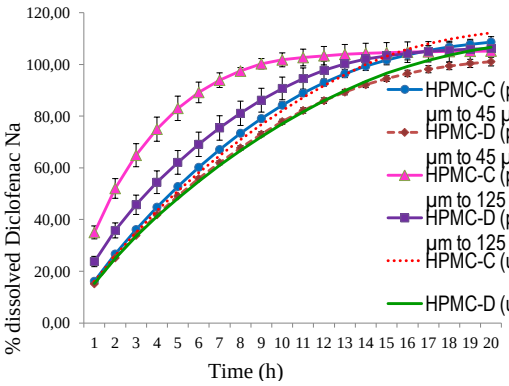
➢ *spheric particles*

- NIR spectra and PLS calibrating models for prediction of particle size of known source
- Prediction of Diclofenac sodium dissolution rate: on the basis of known degree of substitution and source of Hypromellose

Characterization of physicochemical properties of HPMC type 2208 and their influence on the prolonged drug release from matrix tablets; JPBA 2012 (66): 136-143

Results… considering 3rd Hypothesis

- Partile size influence on Diclofenac sodium dissolution rate



The fastest dissolution with HPMC particles 100-125 µm

The slowest dissolution with HPMC particles of non-sieved fractions and fractions 32 -45 µm

"Burst" effect

Statistically different dissolution profiles of particles 100 -125 µm ➡ f_2=45

Characterization of physicochemical properties of HPMC type 2208 and their influence on the prolonged drug release from matrix tablets; JPBA 2012 (66): 136-143

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Živeti zdravo življenje.

FRC sections of specific monographies
≠
Just additional tests

Essentially contribute to proper understanding, which FRC characteristics are important for intended use and/or application.

Discovering key principles in search for new FRC.

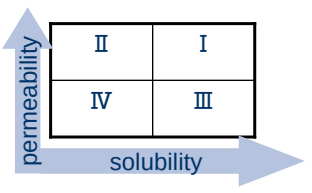
Thank you for your attention!

Aqoat (HPMC-AS)
Solid dispersions by
Spray drying / Hot melt extrusion

Dr. Jörg Brunemann
Harke Pharma

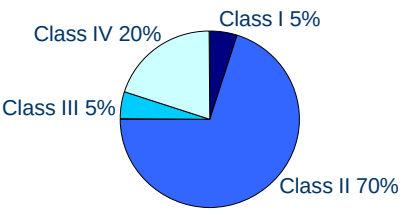
There is a Need for Solubility Enhancement !

Biopharmaceutical Classification System



Class I: **High solubility and high GI absorption**
Class II: **Low solubility and high GI absorption**
Class III: **High solubility and low GI absorption**
Class IV: **Low solubility and low GI absorption**

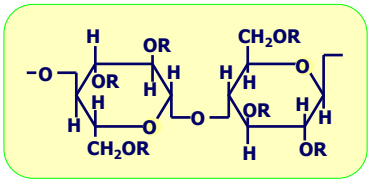
BCS category



Shin-Etsu AQOAT

AQOAT® = Hypromellose Acetate Succinate (HPMCAS)

- ➢ Enteric coating agent
- ➢ Solid dispersion carrier



CAS; 71138-97-1, listed in JPE, USP/NF

AQOAT

Grades of Shin-Etsu AQOAT®

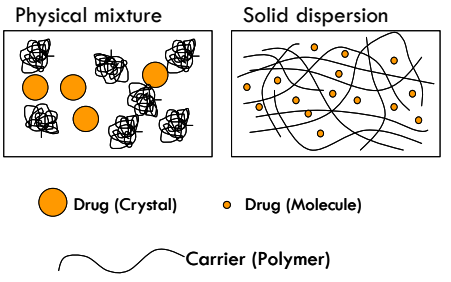
wt (%)	MeO	HPO	Acetyl	Succinoyl	Dissolve at
AS-L	20-24	5-9	5-9	14-18	pH5.5≤
AS-M	21-25	5-9	7-11	10-14	pH6.0 ≤
AS-H	22-26	1-10	10-14	4-8	pH6.8 ≤

particle size: G Type : 1000µm, F Type : 5µm
under development: medium particle size type (for HME)

Approach to Improve drug solubility

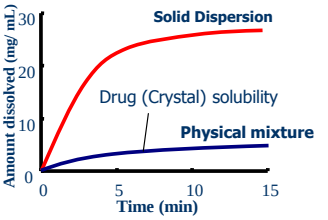
- Physical modifications
  - Micronization
  - Complexation (Surfactants, CyD, etc.)
  - Polymorphs
  - **Solid dispersion** (amorphous)
- Chemical modification
  - Soluble prodrugs
  - Salts

Solid Dispersion: Concept

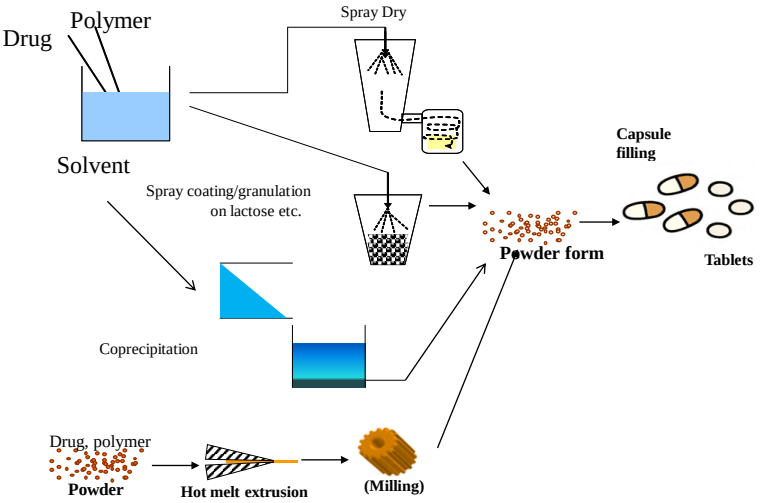


Solid dispersion is a molecular matrix of polymer and drug that achieves significantly greater solubility than the drug alone.

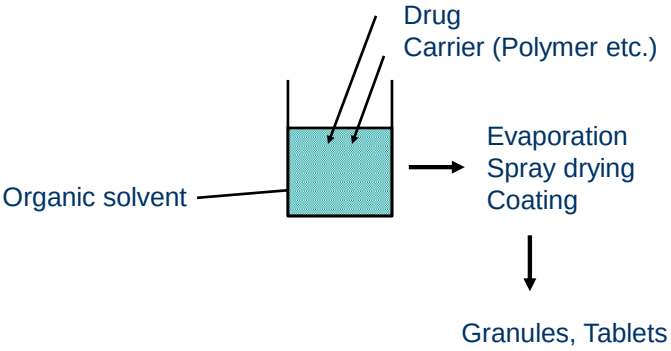
drug	crystal	amorphous
solubility	low	high
stability	stable	unstable (re-crystallization)



Technologies for solid dispersion



Solvent Method / Spray drying, Coating



Drug and polymer are dissolved together in a common solvent, the solution is spray dried or sprayed on a core (spray coating) .

Requirements...

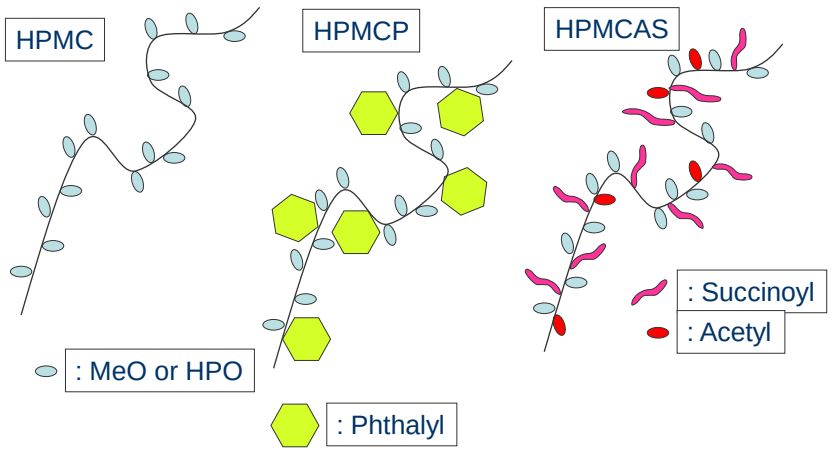
- Significant solubility increase
- Possibly small amount of carrier
- Stable formulation
- Long term inhibition of recrystallization!

Carrier substances for solid dispersion

- Polyvinylpyrrolidone
- Polyethylene glycols
- Cellulose derivatives
  -HPMC, HPC, HPMCP, **HPMCAS**…
- Polyacrylates
- Urea
- New: PVA

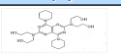
Using HPMC-AS an amorphous polymer chain network is possible!
Cellulose derivatives with bulky side groups were often
more effective compared to the synthetic polymers

Structure



Screening study

- Model drugs

Nifedipine	Griseofulvin	Dipyridamole
 MW: 346	 MW: 352	 MW: 504
water solubility: 0.0063mg/mL	water solubility: 0.0096mg/mL	water solubility: 0.004mg/mL
solubility at pH6.8: 0.0060mg/mL	solubility at pH6.8: 0.0084mg/mL	solubility at pH6.8: 0.0037mg/mL
solubility at pH1.2: 0.0061mg/mL	solubility at pH1.2: 0.0011mg/mL	solubility at pH1.2: >2.5mg/mL
NP	GRF	DIP

Screening study

Model drugs and polymer were dissolved in a mixed solvent (ethanol:dichloromethane = 1:1) and then dried and grinded.

Carrier

- HPMCAS (AQOAT®, AS-M) soluble at pH6.0 ≤
- HPMCP (Hypromellose Phtalate) soluble at pH5.5 ≤
- HPMC (Hypromellose, Pharmacoat 606 water soluble
- PVP (Polyvinylpyrrolidone, K-30) water soluble
- Eudragit L (Poly (MA-MMA) 1:1) soluble at pH6.0≤
- Eudragit E (Poly (BM-DAM-MMA) 1:2:1) soluble at pH5.0≥

Solid dispersion by using AQOAT…

➢ protect drug in the gastric fluid

➢ release drug in the small intestine

Preparation of solid dispersions

- Condition
  Drug:carrier =1:2 (w/w)* *: GRF:carrier = 1:4 (w/w)
  co-solvent : EtOH/$MeCl_2$ (1:1 w/w)
  sprayed onto the Teflon sheet at 50-60˚C and pulverized

Evaluation of solid dispersion

- X-ray diffraction: JP Powder method
- Dissolution test (JP 14th)
  - simulated gastric fluid (pH1.2)
  - simulated intestinal fluid (pH6.8)
  - paddle method, 900mL, 37˚C

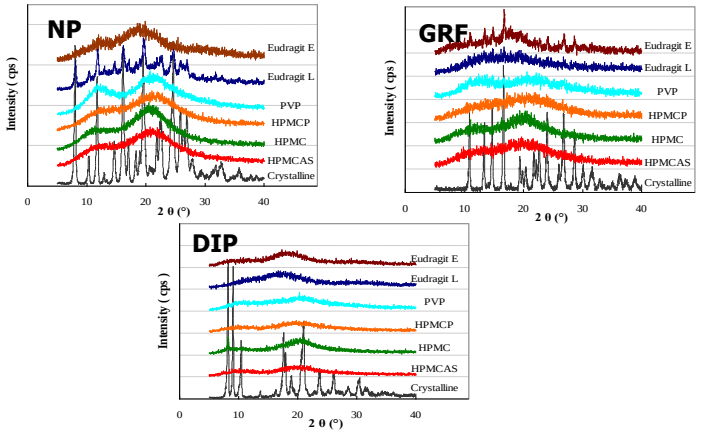
Sample: Solid dispersion containing 5 mg of drug

Buffer: 200 ml of phosphate buffer solution at pH 6.8, 37ºC

Assay: UV at 325 nm (filtration with 0.45 µm)

X-ray diffraction patterns

X-ray diffraction spectra of solid dispersions

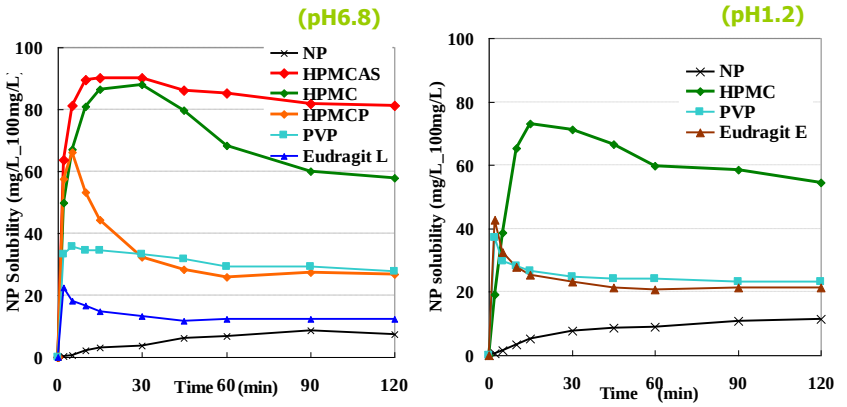


Cellulosic polymers (**HPMCAS, HPMC, HPMCP**) **: amorphous solid dispersion**

PVP, Eudragit L, Eudragit E : partly amorphous solid dispersion

Improvement of drug dissolution

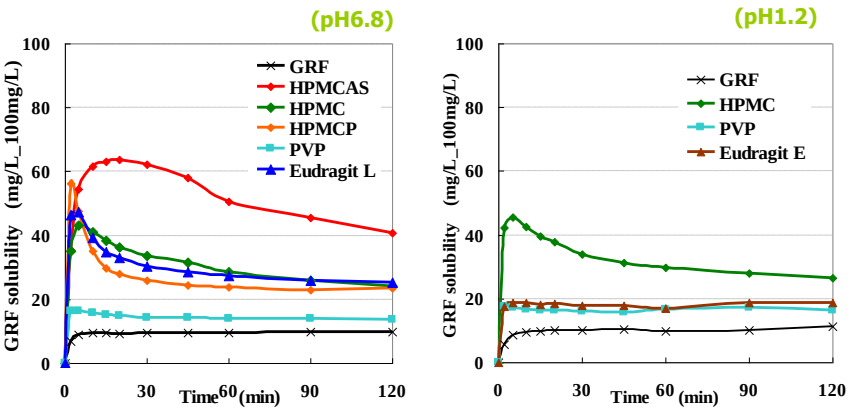
NP (Nifedipine) solid dispersions



Improve: HPMCAS > HPMC> HPMCP> PVP, Eudragit E> Eudragit L

Improvement of drug dissolution

GRF (Griseofulvine) solid dispersions

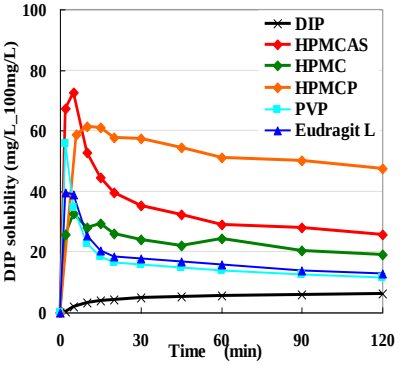


Improve: HPMCAS > HPMC, Eudragit L, HPMCP> PVP, Eudragit E

Improvement of drug dissolution

DIP (Dipyridamole) solid dispersions

(pH6.8)



(pH1.2)

Not necessary to improve solubility

DIP is very soluble at pH1.2; >2.5mg/L (DIP alone)

Improve: HPMCP > HPMCAS > HPMC> PVP, Eudragit L

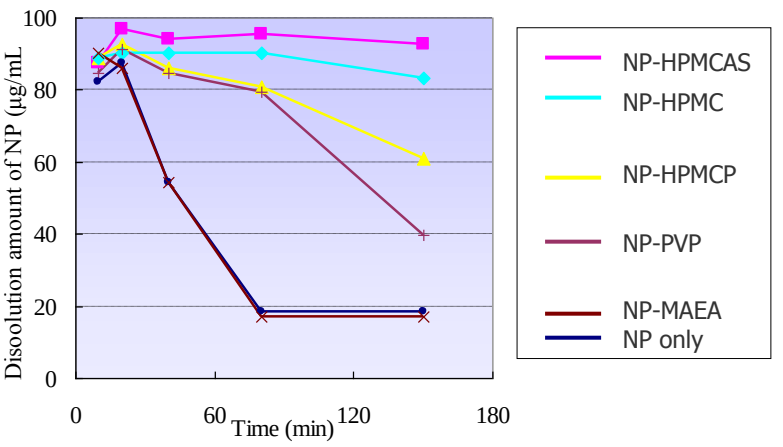
Result ---Screening study

In most cases, AQOAT showed
high improvement
as a carrier for solid dispersion.

Recrystallization Test

Nifedipine:	NP was dissolved in methanol (50 mg in 2 ml)
Buffer:	Each 50 mg of polymers was previously dissolved in a buffer solution (pH 6.8) (50 mg in 500 ml)
PVP:	100 mg,
Acrylic:	150 mg.
Condition:	USP dissolution test apparatus, paddle at 150 rpm, 37 ºC

Recrystallization



Storage stability / drug content

NP content of solid dispersion

Assay by HPLC		Storage period (18M)	
(%)	Initial	40˚C, 75%RH	50˚C, closed bottle
NP	100	-	-
AQOAT	95.6	98.0	96.9
HPMC	100	96.8	98.7
HPMCP	93.1	93.8	93.1
PVP	93.2	93.3	88.4
Eudragit L	94.8	96.2	94.8

In most cases, drug content remain the same level.

Storage stability / solubility

Dissolution of NP after 15 min at pH 6.8.

(mg/L _100mg/,L)	Initial	Storage period (18M)	
		40˚C, 75%RH	50˚C, closed bottle
NP	5	-	-
AQOAT	87	51	89
HPMC	73	36	61
HPMCP	44	10	48
PVP	34	12	34
Eudragit L	20	18	19

50˚C, in the closed bottle (18M) : stable
40˚C, 75%RH (18M): Improvement of drug dissolution was decreased

Aqoat has a low hygroscopicity , the solubility could be maintained

Conclusion

Shin-Etsu AQOAT® exhibited excellent performance as a carrier in solid dispersions.

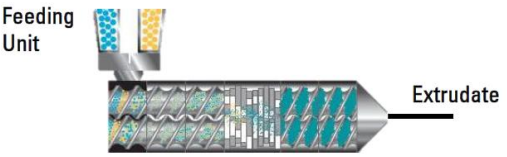
Shin-Etsu AQOAT® showed the greatest level of the improvement of drug dissolution.
It was required small polymer ratio for amorphous drug.

Shin-Etsu AQOAT® suppressed recrystallization of the drug from a supersaturated solution.

Hot melt extrusion

Solid Dispersion by HME

- Solvent free, continuous process
- API and polymer homogenized through extrusion



- Downstream of SD important step
- Maag Automatic provides solutions for pelletizing of extrudates

What is HME ?

- A blended powder consisting of a drug and a polymer are extruded with heat and shearing and the extrudates are milled.
- It is a co melt process
- Limited applicability to heat sensitive drugs and polymers
- Suitable polymers should have a gap between thermal gelation and decomposition temperature.

Aqoat for HME

- Aqoat has a thermal gelation temperature of 120-130°C
- **HPMCAS(AS-L) 120**
- **HPMCAS(AS-M) 130**
- **HPMCAS(AS-H) 135 (seldomly used)**
- Aqoat has a decomposition temperature of about 200 °C
- Recommended extrusion temperature: 140-170°C
- **Medium particle size types (70-300 µm) for HME under development. These grades are recommendable to achieve a uniform blending and constant feed rate.**

Solid Dispersion [HME]: Release of free Acids

HARKE Pharma

Chemical change of HPMCAS

grade	lot	viscosity	moisture (%)		ash	YI	Substituent (%)				free acid		Total acid
AS-MF	8053095	cP	LOD	KF	%		MeO	HPO	Ac	Suc	Succinic acid	Acetic acid	
Temp. (℃)	roter (rpm)												
before HME		2.76	1.3	1.3	0.05	11.8	23.0	7.2	9.3	11.4	0.03	0.04	0.07
160	100	2.66	1.3	1.3	0.08	30.9	22.9	7.1	9.4	11.1	0.44	0.10	0.53
	200	2.60	1.1	1.1	0.04	38.3	23.1	7.2	9.3	10.8	0.68	0.12	0.80
	300	2.60	1.1	1.0	0.07	47.5	23.0	7.1	9.4	10.7	0.85	0.14	1.00
180	100	2.62	1.2	1.2	0.06	32.9	23.0	7.3	9.2	10.8	0.72	0.11	0.82
	200	2.59	1.1	1.1	0.06	35.6	23.0	7.2	9.3	10.8	0.77	0.12	0.89
	300	2.59	1.1	1.0	0.05	46.5	23.1	7.2	9.3	10.9	0.88	0.12	1.00
200	100	2.50	1.1	1.0	0.04	37.6	23.0	7.2	9.2	10.4	1.19	0.16	1.35
	200	2.46	1.0	0.9	0.06	35.5	23.0	7.2	9.3	10.5	1.09	0.15	1.23
	300	2.50	1.2	1.1	0.05	44.1	23.0	7.2	9.1	10.1	1.13	0.16	1.29

Aqoat for HME

HARKE Pharma

Stability for Melt Extrusion

Discussion:

-Cleavage of Succinoyl Groups → Free acid increase
(dissolution pH shifts to higher)

-Color Change (more yellowish)

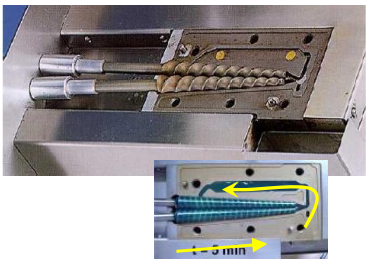
-Slight Reduction in Molecular weight at 200°C

→Possibility of interaction between API and free acid

Case 1: Nifedipine

- **Equipment: Haake MiniLab®**
- **API: Nifedipine**
- **Polymer: HPMCAS (AS-LF, MF, HF), Kollidon VA64**
- **API:Polymer Ratio = 1:2**
- **Extrusion Conditions**

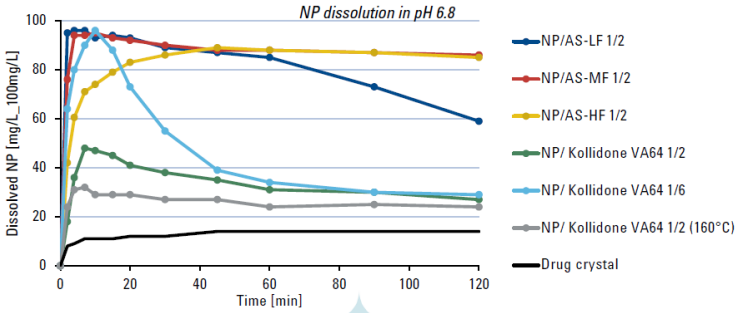
• Temp	150, 160, 170°C
• Screw Length	110mm
• Kneading time	5min
• Extrusion speed	20rpm
• Die	5*3 mm
• Milling: Wonder Blender®	



Case 1: Nifedipine

Hot Melt Extrusion

– Improvement of Drug Dissolution



Improvement (sustained high drug release): HPMCAS > Kollidone VA64

Design of experiments- optimum process parameters

Formulation:
Shin-Etsu Aqoat HPMCAS-MG: Ibuprofen 2:1

Pharma 11 Thermo Scientific, Germany)

						Temperatures (Degrees)								
	Torque (%)	Speed(rpm)	Feeder(kg/hr)	Pressure(bar)	Melt Temperature (Degrees)	Initial	Zone 2	Zone 3	Zone 4	Zone 5	Zone 6	Zone 7	Zone 8	Die
1	16	150	0.15	3	130	20	50	90	100	130	130	130	130	130
2	17	200	0.15	3	131	20	50	90	100	130	130	130	130	130
3	18	200	0.15	10	121	20	50	90	100	120	120	120	120	120
4	22	200	0.30	13	122	20	50	90	100	120	120	120	120	120
5	22	200	0.30	17	111	20	60	100	110	110	110	110	110	109
6	24	300	0.50	20	112	20	60	100	110	110	110	110	110	109
7	24	300	0.50	22	102	20	60	100	100	100	100	100	100	100
8	27	300	0.60	28	103	20	60	100	100	100	100	100	100	100

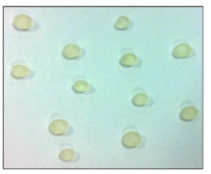
Case 2 Ibuprofen

Extrudates

During processing the measured extruder torque was 25%.

Our general recommendation is to use extrusion temperature for HPMCAS is 150ºC but in the presence of 33% ibuprofen it could be readily processed at 100ºC.

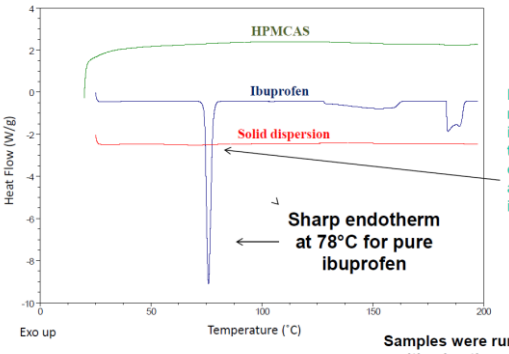
This suggests that ibuprofen acted as a plasticiser and allowed processing at a reduced temperature.



Extruded pellets

Dr. Jörg Brunemann, University of Ljubljana, June 14th, 2018
© HARKE Pharma GmbH

Differential scanning calorimetry study



Disappearance of the melting endotherm of ibuprofen (at 78°C) in the solid dispersion confirmed the amorphous state of ibuprofen

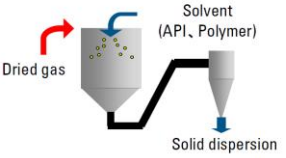
Samples were run from 25°C to 200°C with a heating rate of 10°C per min

Manufacture of Solid Dispersin

ShinEtsu

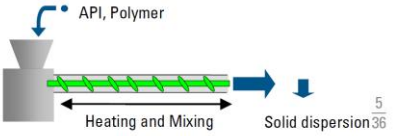
Spray Dryer Method

- Need Organic solvent
- Need drying process
- Often need granulation
- Organic solvent should dissolve API and polymer



Hot Melt Extrusion Method

- **No need organic solvent**
- **Less manufacture process**
- **Only milling**
- **Continuous process**
- Some API and polymer cannot use if decomposition temp. and glass transition temp. is not suited



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Many thanks for your attention!

HARKE Pharma GmbH · Xantener Straße 1 · D-45479 Mülheim an der Ruhr · Germany
Phone:+4920830692270 · E-mail: drb@harke.com · Internet: www.harke.com

National Institute of Chemistry, Ljubljana, Slovenia

EUROPEAN UNION
EUROPEAN REGIONAL DEVELOPMENT FUND
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NANOCELLULOSE – BIOMATERIAL OF THE FUTURE

Assoc.prof.dr.Matjaž Kunaver

matjaz.kunaver@ki.si

BIOMASS WASTE – A SOURCE OF RAW MATERIALS AND NANOCELLULOSE

- INTRODUCTION
- SOURCES OF BIOMASS
- BIOMASS COMPONENTS AND THEIR CONVERSION INTO VALUABLE CHEMICALS
- BIOMASS LIQUEFACTION AND UTILIZATION IN DIFFERENT APPLICATIONS
- ENERGY FROM LIQUEFIED BIOMASS
- NANOCELLULOSE AND ITS APPLICATIONS
- CONCLUSIONS

CEEPUS Summer School, 14 June 2018, Ljubljana, Slovenia

Lignocellulose-based materials

Lignocellulose-based materials

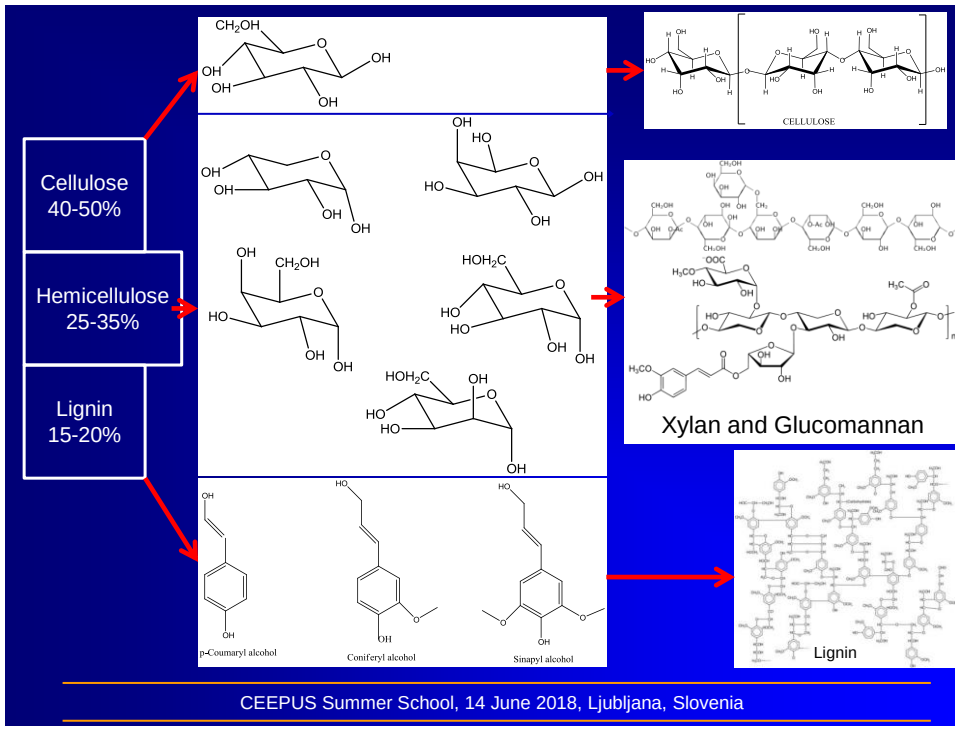
LJUBLJANA: 350.000 inhabitants

5.700 tons of different wood waste materials per year, mainly broken furniture and packaging materials.

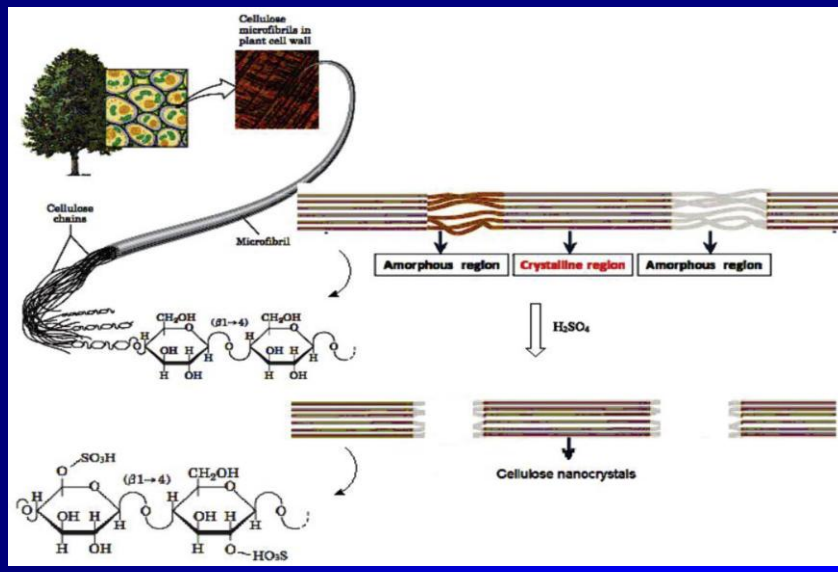
2.300 tons of forest residues are deposited, mainly tree branches, bark and larger pieces of timber

MAIZE: 332 Mt/year – USA
817 Mt/year – world production

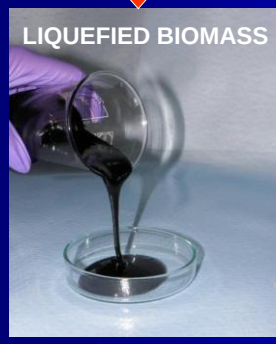
Timber production: 30% of the tree mass is waste



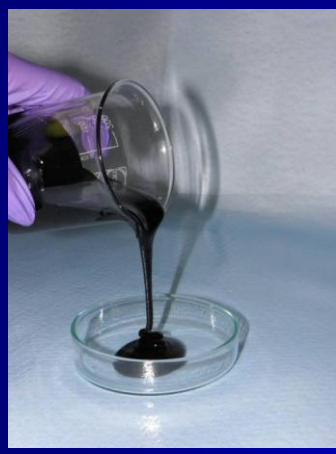
Microstructure of wood microfibrils



Trends in Food Science & Technology, 71, 2018, 268-273



What is liqueifed biomass ?



A blend of depolymerized and solubilized wood components obtained by reaction with polyhydric alcohols in the presence of acid as a catalyst.

Liquefaction converts biomass into a feedstock for the new, environmentally friendly polymers

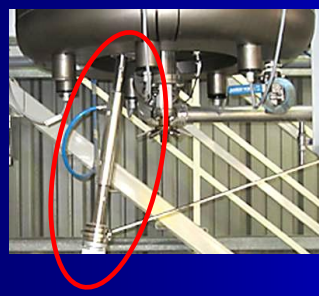
Liquefaction process

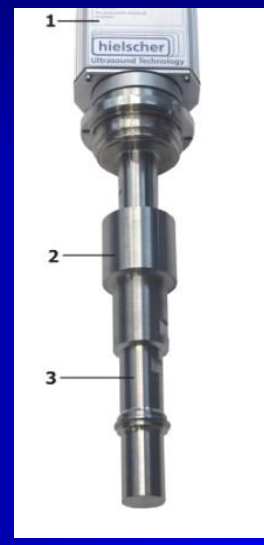
- Glass reactor with external heating, mixing
  - 2 to 3 hours at 150 - 180 °C
- Ultrasound 105W/cm^2
  - 10 – 25 minutes at 150 - 180 °C

- Typical reaction mixture:
  - 100 g wood (or lignocellulosic material)
  - 300 g glycol (glycerol, diethylene glycol)
  - 9 g acid catalyst (pTSA)

Kunaver M, Jasiukaitytė E, Čuk N (2012) Ultrasonically assisted liquefaction of lignocellulosic materials Bioresource Technology 103:360-366

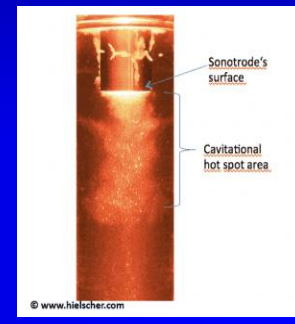
Liquefaction process with ultrasound





SAVES:
~ 30 % of energy,

50% of reaction time



SYNTHESIS OF POLYURETHANE FOAMS

Polyurethane foams based on liquid wood poliols,

Density = 0.03 -0.05 g/ cm^3

Compressive strength at 10% strain: 300-400kPa

Tensile strength: 127 kPa

Thermal conductivity:

0.029 W/mK

Addition of glycerol

ADHESIVES WITH THE ADDITON OF THE LIQUEFIED WOOD - APPLICATIONS

Standard adhesives: Melamine – formaldehyde, melamine – urea – formaldehyde resins

Same or better mechanical properties, formaldehyde emission reduced by 50%

LIQUEFIED BIOMASS – LIQUID FUEL

Storage tank → Preheating (small) boiler → Central heating burner

ADVANTAGES:

HEATING VALUES: 22 -24 MJ/kg

-Liquid

-High energy value

-High biomass and renewable resources content

DRAWBACKS:

- Corrosive (easily avoided with affordable materials)

- Sulphur (1/2 allowable content for L.h. Oil)

- Ash (under investigation)

Fundamental question: Is economy really OK?

1. ADHESIVES for particleboards: 0.63 EUR/kg

   Addition of 30% of liquefied biomass: 0.53 EUR/kg

Formaldehyde release: less than 5mg/100g

2. FUEL price for production of 10kWh energy:

   Propane/Butane gas: 1.18 EUR

   Natural gas: 0.80 EUR

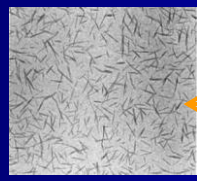
   Light heating oil: 1.41 EUR

   Liquefied biomass: 0.98 EUR

Why nanocellulose (NCC):

- The most abundand natural polymer, biodegradable.
- Low density, high aspect ratio, high surface area.
- Filler in nanocomposites with excellent mechanical properties.
- Filler in green composites.
- Applications in food and pharmaceutical industry.
- Wide variety of the surface modifications

NCC is usually produced from native cellulose sources by isolation of its crystalline regions – the amorphous regions are hydrolyzed and degraded into soluble products.



Controlled hydrolysis by sulfuric acid – typically 65 wt%.

Source: Brinchi *et al*.: Carbohydrate Polymers 94 (2013) 154– 169

VARIOUS PROCESSES OF EXTRACTING NANOCELLULOSE FIBERS:

- MECHANICAL TREATMENT (CRYOCRUSHING, GRINDING)
- HIGH PRESSURE HOMOGENIZING
- CHEMICAL TREATMENTS – ACID HYDROLYSIS
- BIOLOGICAL TREATMENT – ENCYME-ASSISTED HYDROLYSIS
- TEMPO – OXIDATION ON SURFACE AND MILD MECHANICAL TREATMENT
- SYNTHETIC AND ELECTROSPINNING METHODS
- ULTRASONIC TECHNIQUE

(Source: Carbohydrate polymers 87, (2012), 963-979)

DEPOLYMERIZATION WITH GLYCOL

- Cellulose source (wood, cotton, paper, etc.),
- Glycol: ethylene glycol, diethylene glycol, PEG 400, etc.,
- Methane sulfonic acid (3 wt% on glycol amount),
- Glas reactor with external heater,
- 120 to 180 min at 140 – 160^{o}C
- Product centrifugation and washing with 1,4-Dioxane or any other medium polar solvent,
- The product: NCC suspension in solvent

- OPTION: use of ultrasound as an additional energy source: 4 times shorter reaction time

Kunaver M, Anžlovar A, Žagar E (2016) The fast and effective isolation of nanocellulose from selected cellulosic feedstocks Carbohydrate polymers 148:251-256

New method for NCC isolation

Milling, cutting

Depolymerisation with glycols

3 -5 times

Centrifugation, rinsing with solvents

Bleaching

NCC suspension in water

NCC suspension in organic solvent

1st STEP: LIQUEFACTION WITH GLYCOLS – PILOT PLANT REACTOR

FORMULATION:

- glycols (ethyileneglycol, glycerin –from biodiesel production)
- 3% methanesulfonic acid
- milled biomass

Heating and mixing for 2 to 3 hours at 150°C.

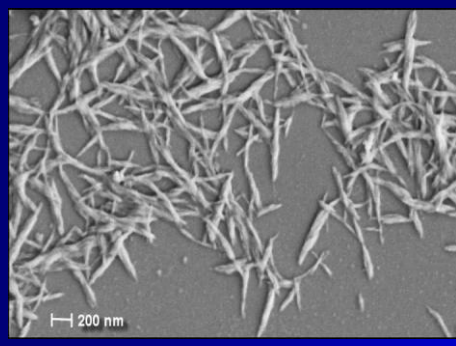
2nd STEP: CENTRIFUGATION AND WASHING

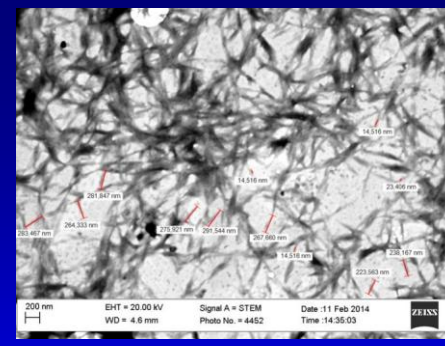
PILOT CENTRIFUGE:

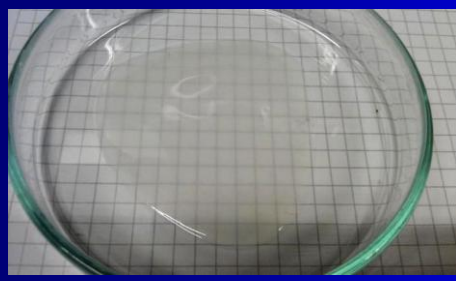
16 liter capacity

UTILIZATION OF ULTRASOUND:

- 50% shorter reaction time
- Breaks down aggregates and makes a stable suspension



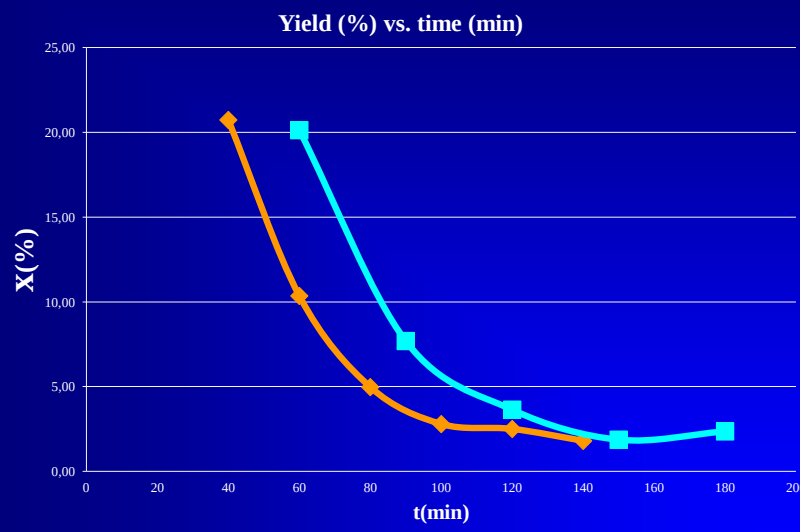




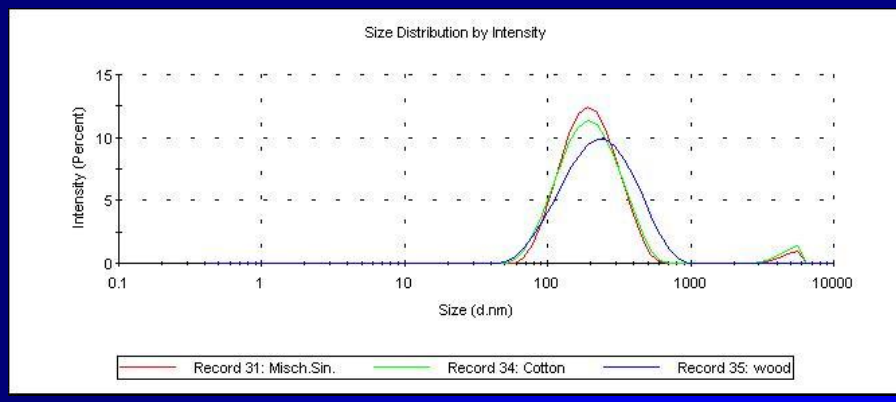
NCC properties from different sources:

Biomass	NCC recovery (%)	Crystallinity index (C_rI)	Average NCC crystal length (nm)	Average NCC crystal width (nm)
Cotton linter	74.5±6.0	89%	242±8.0	12.7+/-0.4
Spruce wood	61.5±3.2	68%	235±23.6	6.3+/-0.1
Chinese silver grass	55.6±4.0	80%	203±13.8	6.8+/-0.2
Eucalyptus wood	63.0±8.5	79%	273±17.3	7.3+/-0.1

Utilization of ultrasound

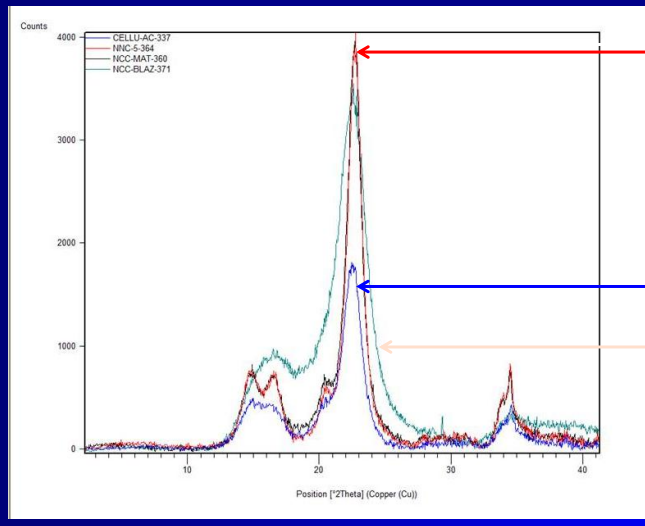


DYNAMIC LIGHT SCATTERING



DLS hydrodynamic size distributions of representative samples

Crystallinity index:



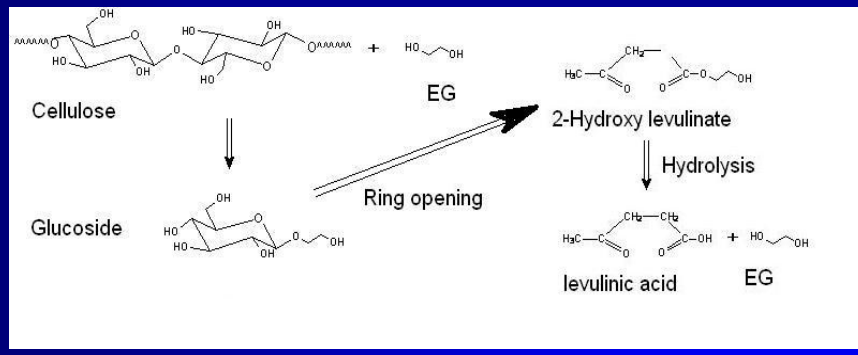
NCC – GLYCOL DEPOLYMERIZATION

MCC

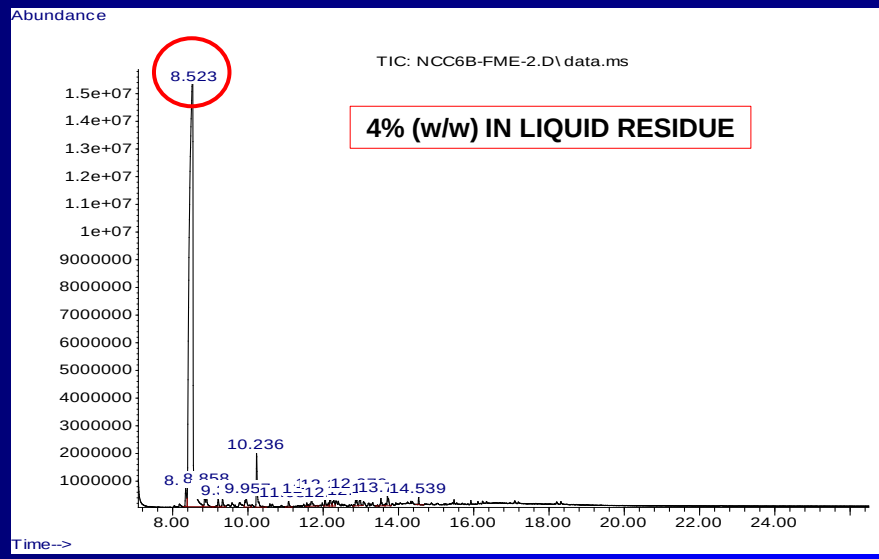
COMMERCIAL SAMPLE OF NCC

SIDE PRODUCTS:

•**Polysaccharides → glucoside→ levulinic ester**

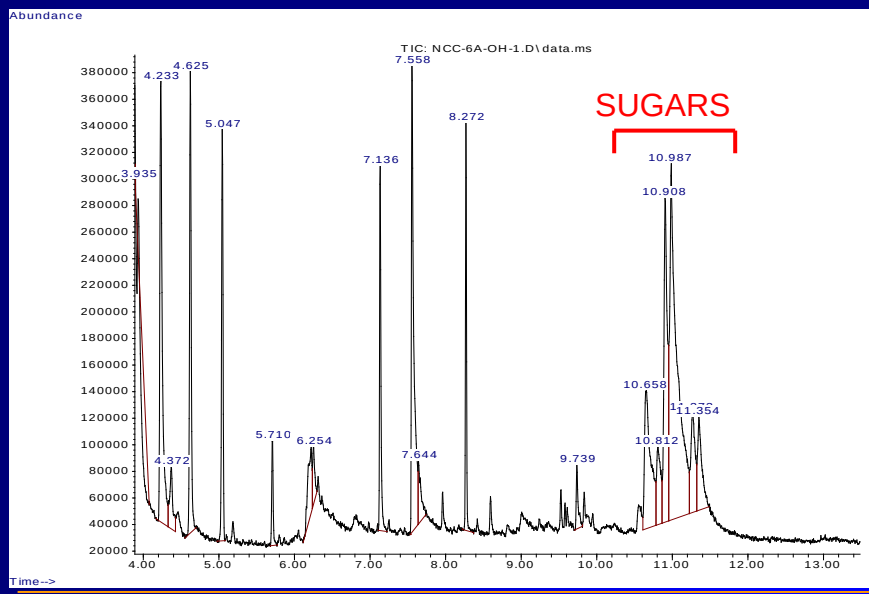


SIDE PRODUCTS: Levulinic acid (CH_3-CO-CH_2-CH_2-COOH)



4% (w/w) IN LIQUID RESIDUE

SIDE PRODUCTS: Sugars

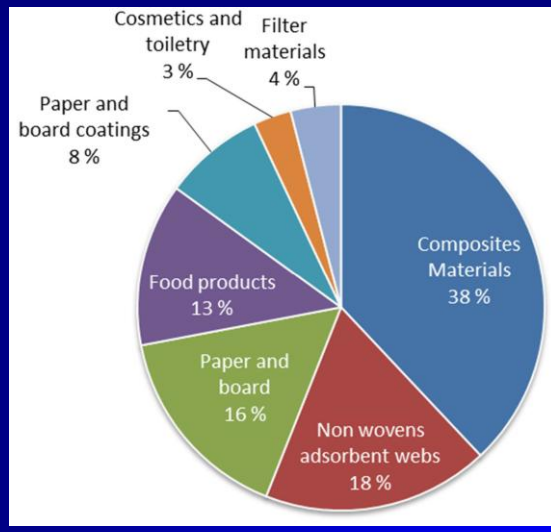


APPLICATIONS and MARKET PRICES

Blue Goose Biorefineries

Slovenian pilot lant production: 300 -400 EUR/kg of suspension (6- 10% in water), capacity: 10 kg/day

NUMBER OF PATENTS BY MARKET SEGMENTS

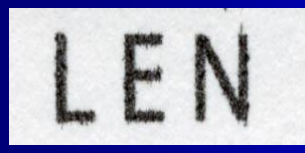


APPLICATIONS

Paper chemicals	Paper coatings	Better printability
Coatings industry	Coatings	Mechanical properties, Thixotropy, viscosity modifications
Packaging industry	Food packaging foils	Better barrier properties
Polymers	All kind of polymer composites	Better mechanical properties
Pharmacy	Drug carriers	Tissue scaffolding, drug delivery
Construction materials	Beton	Flexural strength (+30%)
Cosmetics	Filler	
Electronics	Flexible circuits	

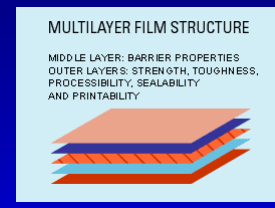
APPLICATIONS

to study and develop the propper structure of the starch and PVA coating with the optimal added concentration of the NCC and thus to improve the printability of the paper. results of the measurements have shown, that the coatings have improved the mechanical properties of the samples, by which the printability of the sample paper has also improved.



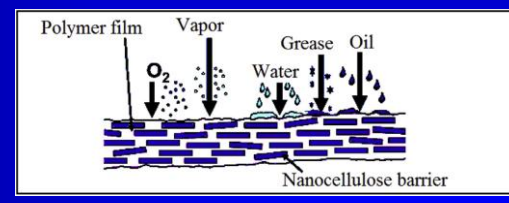
MEDVEŠEK, Sabina. *Influence of nanocrystallized cellulose on paper printability : master's thesis*. University of Ljubljana, 2017. https://repozitorij.unilj.si/IzpisGradiva.php?id=91471&lang=slv.

NANOCELLULOSE IN PACKAGING INDUSTRY



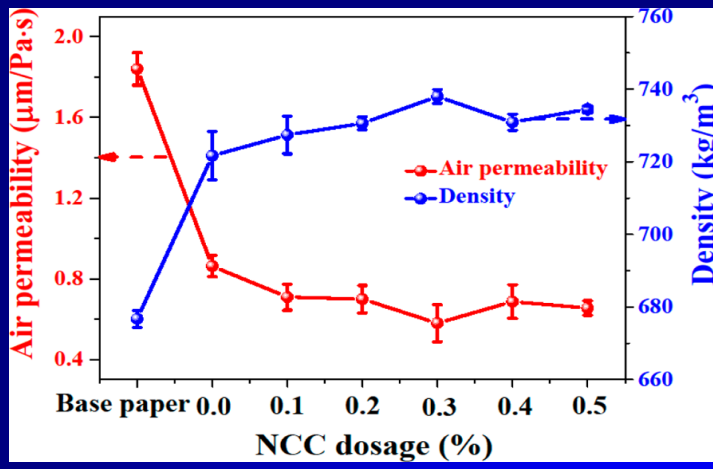
Fiber based packages
- Strength additive
- Binder
- Barrier

Plastic packaging
- Reinforcement
- Barrier
- Part of multilayer structures



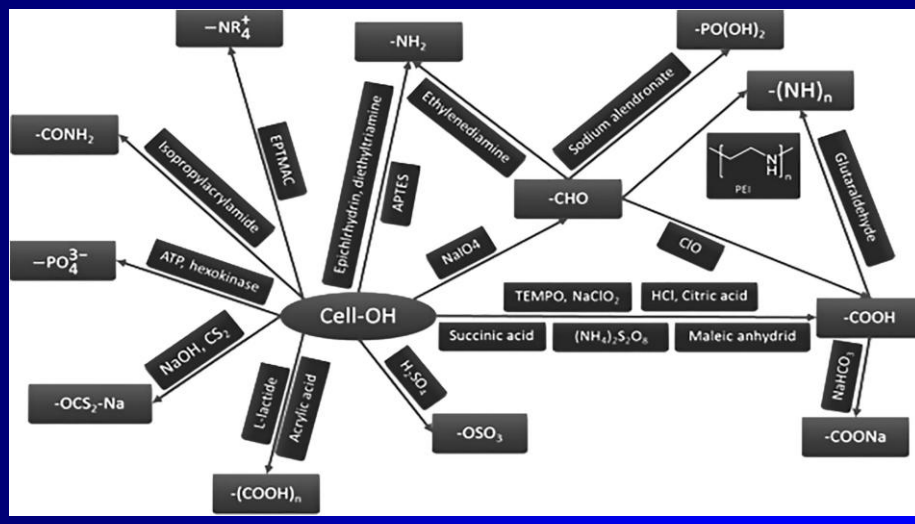
Nair, S. Et.All., Sustain. Chem. Process., 2014, 2, 23.

NANOCELLULOSE IN PACKAGING INDUSTRY



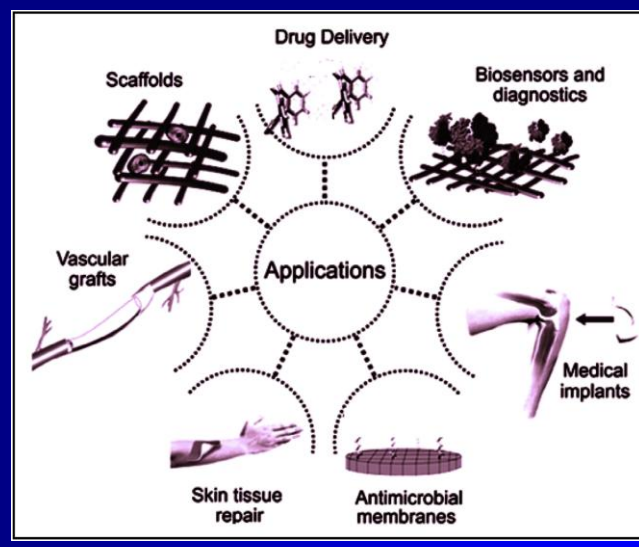
Shujie Yang *Et.Al.*:Surface Treatment of Cellulosic Paper with Starch-Based Composites Reinforced with Nanocrystalline CelluloseInd. Eng. Chem. Res. 2014, 53, 13980−13988

NANOCELLULOSE MODIFICATIONS



R.K. Mishra et al., Journal of Saudi Chemical Society (2018), Https://doi.org/10.1016/j.jscs.2018.02.005

Biomedical applications of nanocellulose



J. Rojas *et all.*: Cellul. - Fundam. Asp. Curr. Trends, InTech, 2015. doi:10.5772/61334.

A viewpoint on the gastrointestinal fate of cellulose nanocrystals – Koshani R., Madadlou A.: Trends in Food Science & Technology, 71, 2018, 268-2173

- Positively charged nanoparticles do not adhere to the mucus and agglomerate.
- Nanoparticles penetration is hindered due to its size, namely 200 nm.
- Nanoparticles are expected to bind with opsonin proteins and cleared out.

Nanocellulose in biomedicine – Lin N., Dufresne A.: European Polymer Journal, 59, 2014, 302-325

The paper summarizes different aspects of utilization of nanocellulose in medicine:

- NC is biocompatible, invoking only moderate if any body responses *in vivo.*
- The inhalation of plentiful NC may induce pulmonary inflammation.
- No cytotoxic effect on nine different cell lines was determined.
- NC based biomaterials can encourage cells attachment on tissue bioscaffold.
- NC can bind water soluble antibiotics, anticancer agents.

Nanocellulose in biomedicine – Lin N., Dufresne A.: European Polymer Journal, 59, 2014, 302-325

- NC is suitable carrier for the immobilization of enzyme and protein by covalent binding or adsorption.
- Replacement of blood vessels : BAYSIC© bacterial synthesized cellulose – mechanical strength, water retention, low roughness.
- NC wound dressing – chronic wounds – reduction of healing time.
- NC has porous network structure for potential transfer of antibiotics or inorganic antimicrobila agents.

CEEPUS Summer School, 14 June 2018, Ljubljana, Slovenia

NANOCELLULOSE PRODUCTION

CELLULOSE NANOCRYSTALS (CNC) CAPACITY CURRENT AND ANNOUNCED 2015
(kg per day)

CelluForce, Canada	1,000
American Process, U.S.	500
Holmen (Melodea), Sweden *	100
Alberta Innovates, Canada	20
US Forest Products Lab	10
Blue Goose Biorefineries, Canada	10
India Council for Ag. Research	10
FPInnovations, Canada	3
Melodea, Israel	Pilot

http://www.tappinano.org/media/1114/cellulose-nanomaterials-production-state-of-the-industry-dec-2015.pdf

Conclusions

- Lignocellulosic biomass is a natural source of many valuable chemicals.
- The initial step is depolymerisation and derivatization.
- Different reaction pathways lead to different chemicals.
- The liquefaction reaction leads to adhesives, polyesters, polyurethane foams and fuel.
- One of the newest applications is the isolation of nanocellulose from natural sources.

Acknowledgments:

Authors wishes to gratefully acknowledge the support for the presented work received from :

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-RDI project Cel.Cycle: »Potential of biomass for development of advanced materials and bio-based products« (contract number: OP20.00365), co-financed by the Republic of Slovenia, Ministry of Education, Science and Sport and European Union under the European Regional Development Fund, 2016–2020.

-BIOECO-R.D.I. – Program Interreg ADRION (project 605)

KRKA

Living a healthy life.

Polymer Characterization - Analytical Approach in Pharmaceutical Industry

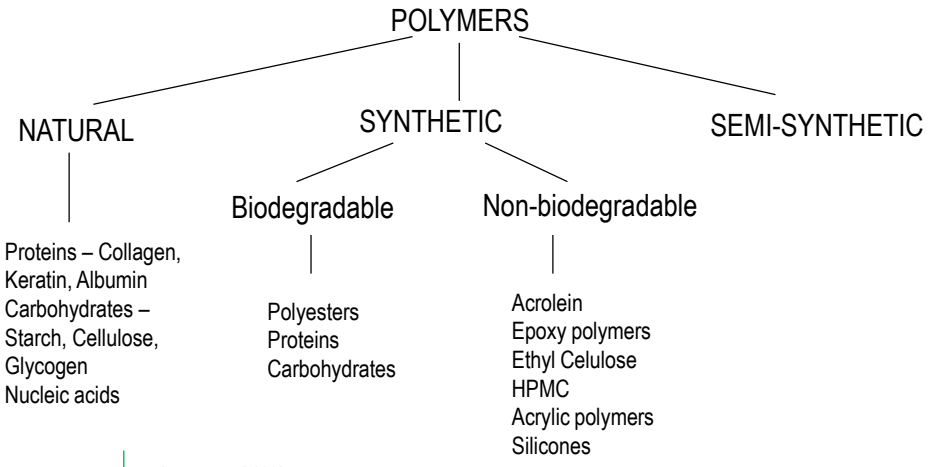
Boštjan Jerman

June 2018

CONTENT

- FIELDS OF USE
- INTRODUCTION OF PHYSICAL, CHEMICAL AND MECHANICAL PROPERTIES
- VARIOUS ANALYTICAL TECHNIQUES USED IN CERTAIN FIELDS
- SPECIAL CASES

CLASSIFICATION – Based on origin



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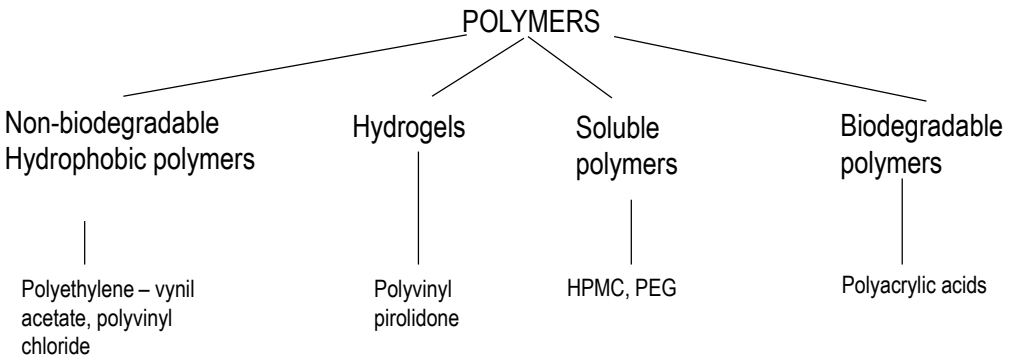
CLASSIFICATION – Based on interaction with water



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Criteria Followed in Polymer Selection

- Should be soluble and easy to synthesize; should have a finite molecular weight.

- Should provide drug attachment and release sites for drug – polymer linkages.

- Should be compatible with biological enviroment, i.e. non-toxic and non-antigenic.

- Should be biodegradable or be eliminated from body after its function is over.

Applications in Conventional Dosage Forms

EXCIPIENTS
- Binders: Cellulose derivatives (MC, HPMC, HEC, HEMC)
- Disintegrating agents: carboxyl methyl cellulose
- To mask unpleasant taste
- Solid Dispersions

LIQUIDS
- Dispersion agents in solids
- Viscosity enhancers (controlling the flow)
- Emulsifying agents (Span, Tween)

Applications in Conventional Dosage Forms

SEMISOLIDS
- Thickening agents (PEG)
- Suppository bases (PEG)
- Gel preparation

ACTIVE INGREDIENTS:
- Some drugs themselves are polymers (insulin, heparin, albumin, laxative methyl cellulose, herbal extracts)

Applications in Conventional Dosage Forms

CAPSULES:
- Gelatine, HPMC

(FILM) COATING MATERIALS:
- cellulose derivatives, acrillyc derivatives (Eudragit)

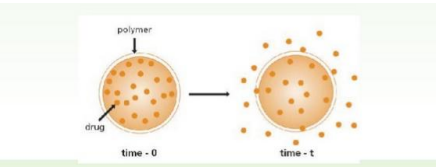
PACKAGING MATERIALS:
(barrier properties)
- PE (HDPE, LDPE), PVDC, PVC
- PP
- PVC

Applications for Modified Release Systems

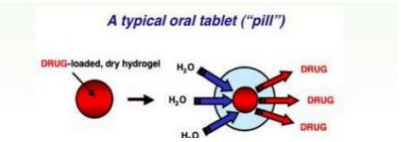
- Reservoir systems
- Matrix systems
- Swelling Systems
- Biodegradable systems
- Nanoparticles
- Solid Dispersions
- Transdermal patches
- Ocular drug delivery

- Polypeptide vesicles for drug delivery
- Micelles for cancer therapeutics

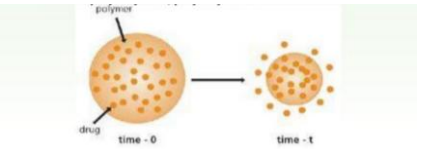
Reservoir Diffusion System



Swelling System

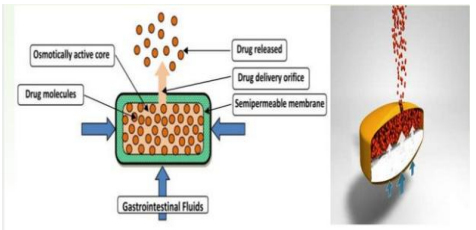


Matrix Diffusion System

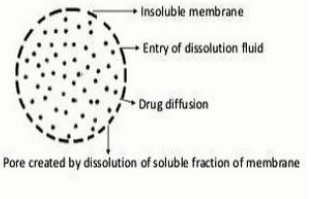


Controlled drug delivery systems

- Osmotically Controlled Drug Delivery
- Diffusion controlled Drug Delivery
- Muco-adhesive Drug Delivery



- Drug encased in a partially soluble membrane.
- Pores are created due to dissolution of parts of membrane.
- It permits entry of aqueous medium into core & drug dissolution.
- Diffusion of dissolved drug out of system.
- Ex- Ethyl cellulose & PVP mixture dissolves in water & create pores of insoluble ethyl cellulose membrane



Physical Properties

Polymers display different thermal, physical, and mechanical properties depending on their structure, molecular weight, linearity, intra- and intermolecular interactions.

Ordered structure: PP

Irregular structure: majority of polymers
Amorphous structure (glass)

Crystallinity increases the barrier properties of the polymer. Small molecules like drugs or solvents usually cannot penetrate or diffuse through crystalline domains. Therefore, crystalline polymers display better barrier properties and durability (packaging materials).

Physical Properties

Diffusion and **solubility** are two important terms that are related to the level of crystallinity in a polymer. Amorphous polymer is preferred when the release of a drug or an active material is intended.

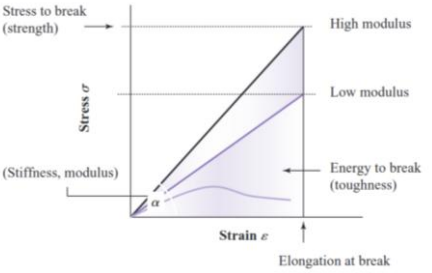
A crystal cell displays different properties along longitudinal and transverse directions. This causes the polymer to behave like an anisotropic material.

The addition of a plasticizer to a polymer results in a reduction in the glass transition temperature of the mixture. Since plasticizers increase molecular motion, drug molecules can diffuse through the plasticized polymer matrix at a higher rate, depending on the plasticizer concentration.

Mechanical properties

Resistance against:
stretching (tensile strength),
compression (compressive strength),
bending (flexural strength),
sudden stress (impact strength) and
dynamic loading (fatigue).

VISCOELASTIC PROPERTIES
Polymers are neither a pure elastic nor a pure fluid material. They have the ability to store energy (elastic behavior) and to dissipate it (viscous behavior).



Polymeric materials such as fibers and highly cross-linked polymers display elastic behavior, in other words, a linear stress/strain correlation up to their breaking point.

Physical Properties– Thermal Analysis

DSC

TGA

TMA

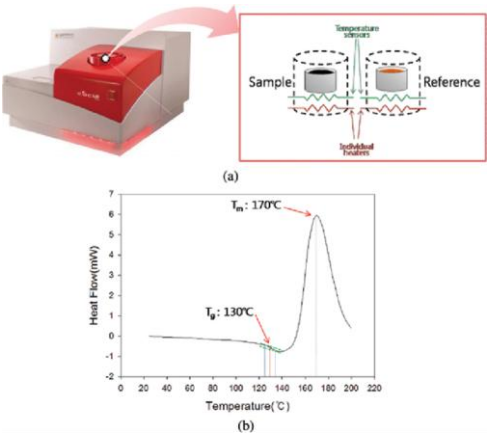
DMA

coupled techniques




Physical Properties– Thermal Analysis

DSC:



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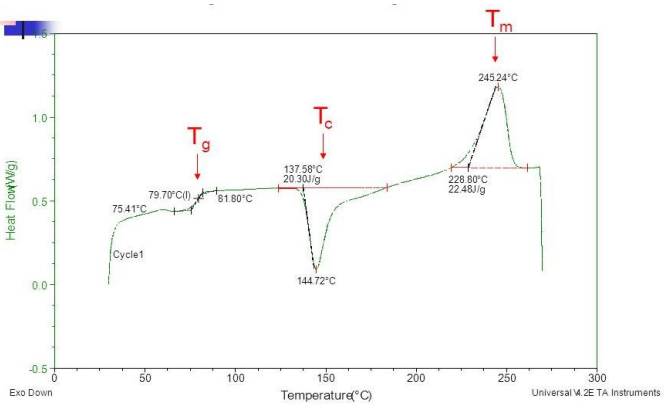
Physical Properties– Thermal Analysis

DSC: phase diagrams, glass transition temperature, melting temperature, degree of crystallinity, heat of fusion/crystallisation, polymer/mix detection, thermal history, decomposition temperatures

THERMALLY INDUCED TRANSITIONS
Melting temperature T_m
Glass transition temperature T_g
((Cold) Crystallisation, Curing, Annealing, Quenching)



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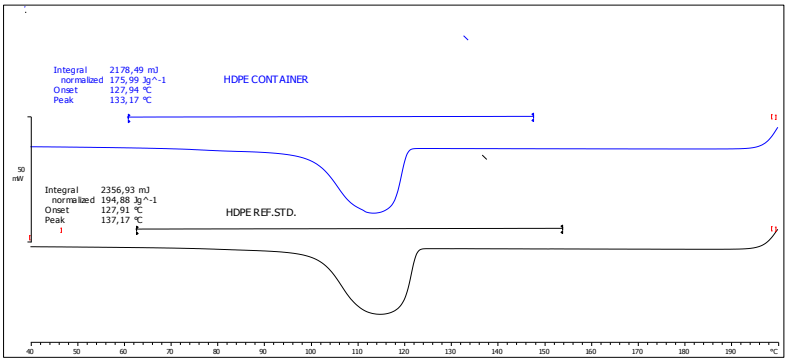
Physical Properties– Thermal Analysis DSC examples

QC release:



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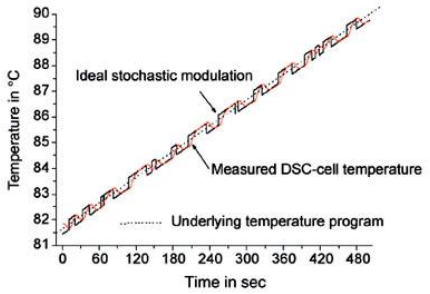
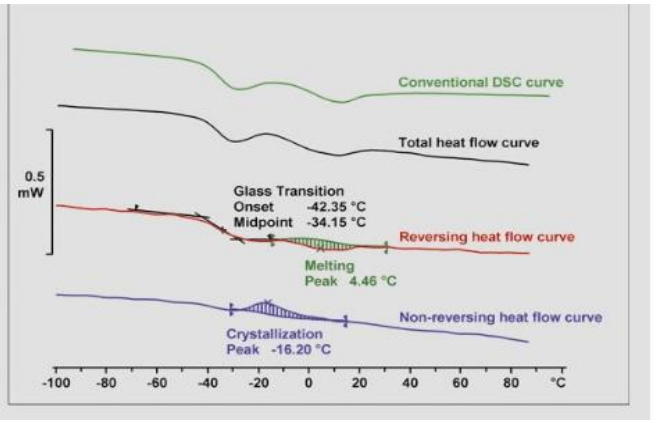
Physical Properties– Thermal Analysis DSC examples

Overlapping effects?
Temperature modulated DSC
(MDSC, TOPEM)





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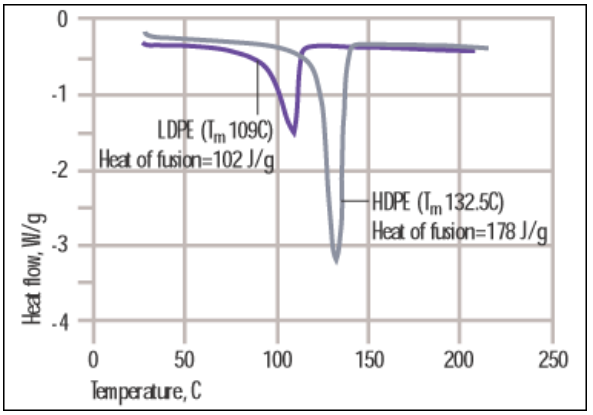
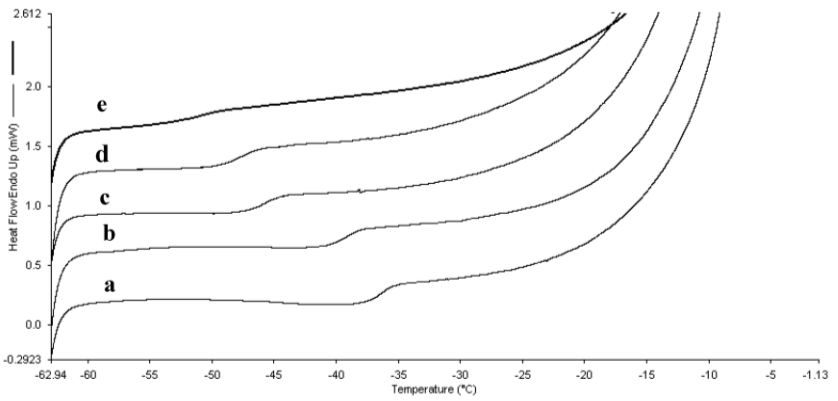
Physical Properties– Thermal Analysis DSC examples

Crystallinity
Purity



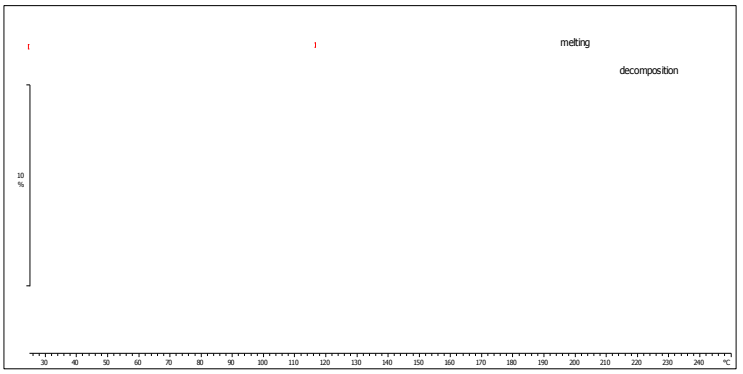
Physical Properties– Thermal Analysis DSC examples

DSC –
Plasticising agents are lowering Tg



Physical Properties – Thermal Analysis

TGA: thermal stability (also oxidative), additive content



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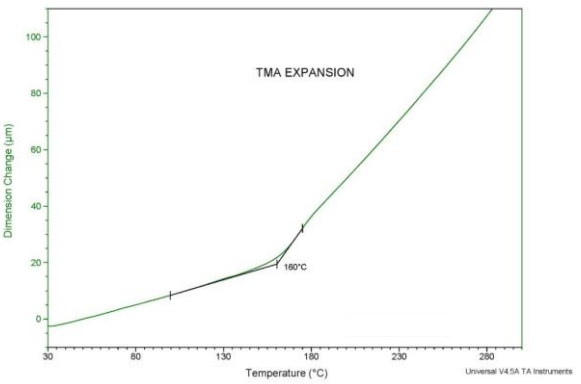
Physical Properties – Thermal Analysis

TMA:
glass transition,
amorphous softening,
linear expansion coefficient



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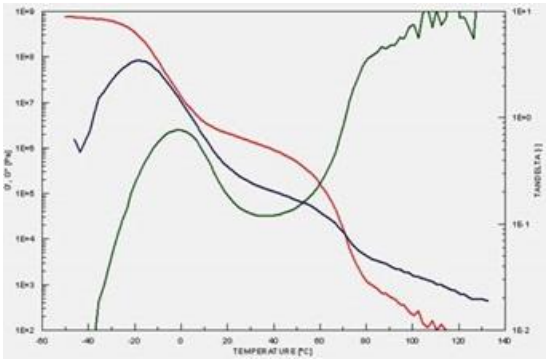
Physical Properties – Thermal Analysis

DMA:
glass transition,
visco-elastic behavior
(elastic modulus, shear modulus)



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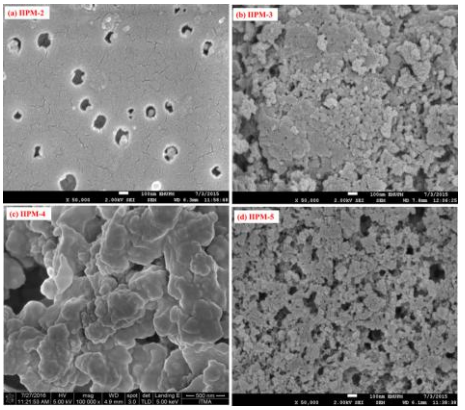
Physical Properties– MORPHOLOGY

SEM
PSD
AFM
SEC



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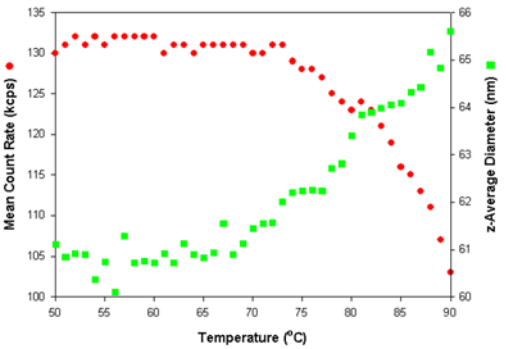
Physical Properties– MORPHOLOGY

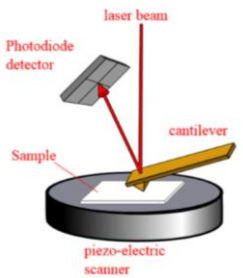
SEM
PSD
AFM
SEC

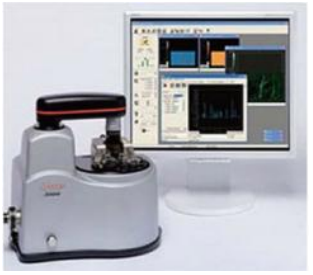
Dynamic light scattering can easily monitor temperature dependent changes in the conformation of polymer particles.

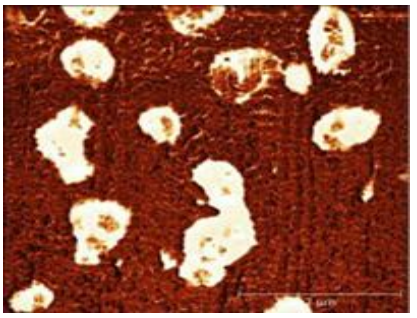


Physical Properties– MORPHOLOGY

SEM
PSD
AFM
SEC

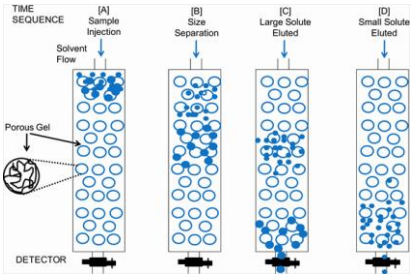


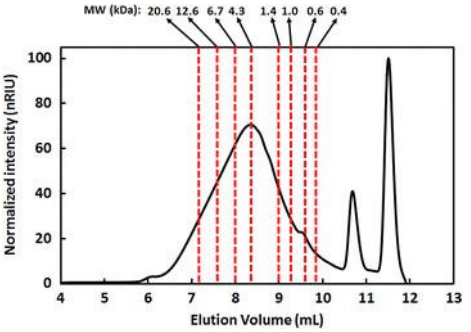




Physical Properties– MORPHOLOGY

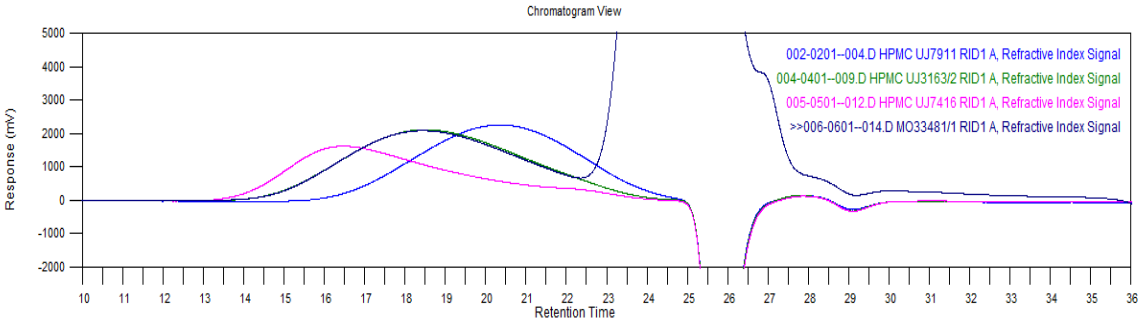
SEM
PSD
AFM
SEC



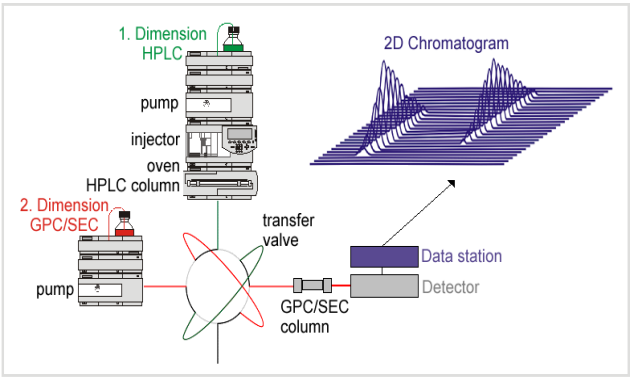


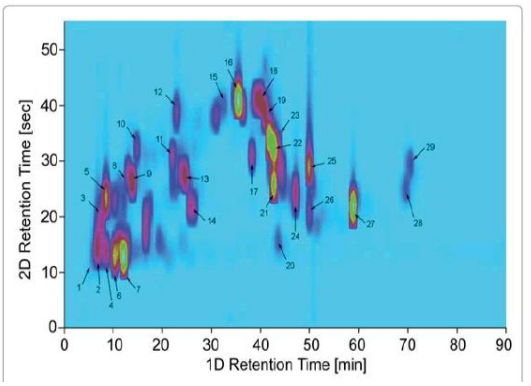
SEC

DEFORMULATION – type of HPMC



2D CHROMATOGRAPHY

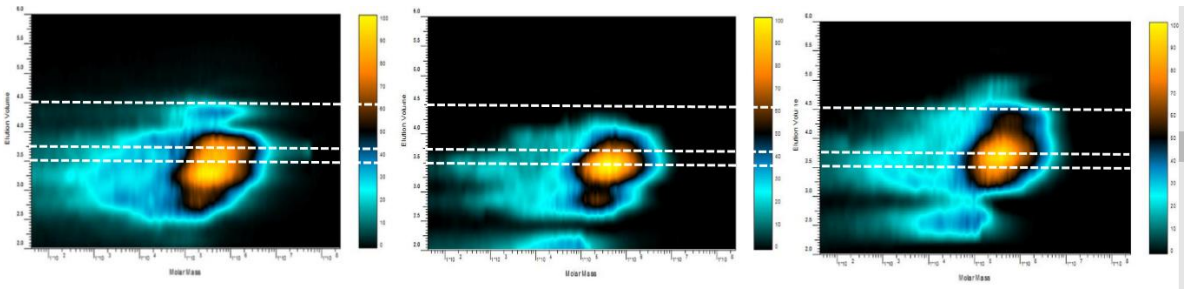




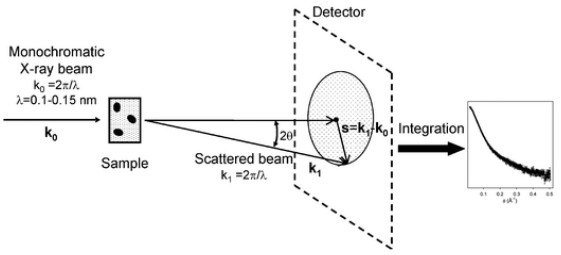
2D CHROMATOGRAPHY

LC – SEC

Degree of of substitution



SMALL ANGLE X-RAY SCATTERING - SAXS



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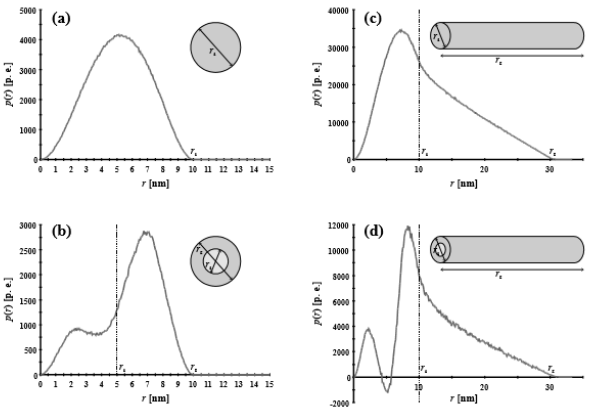
SMALL ANGLE X-RAY SCATTERING - SAXS

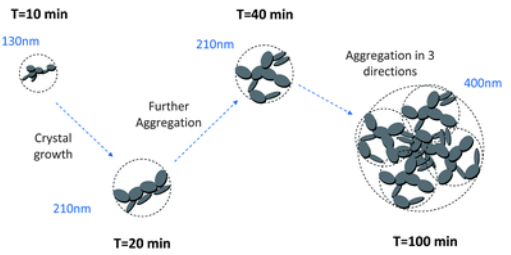
Particle shape modelling



KRKA

SMALL ANGLE X-RAY SCATTERING - SAXS

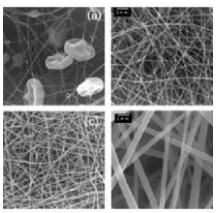
SAXS patterns contain data concerning correlations on an inter-molecular level: necessarily samples where there is macromolecular or aggregate order

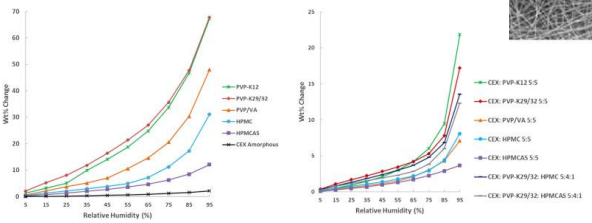


POLYMERS AS EXCIPIENTS

Binders: Cellulose derivatives (MC, HPMC, HEC, HEMC)
Emulsifying agents (Span, Tween)
Thickening agents (PEG)

Disintegrating agents: carboxyl methyl cellulose

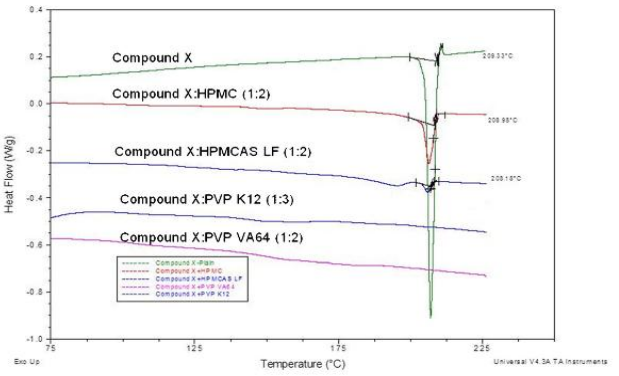




POLYMERS AS EXCIPIENTS

Solid dispersions: hot melt extrusion

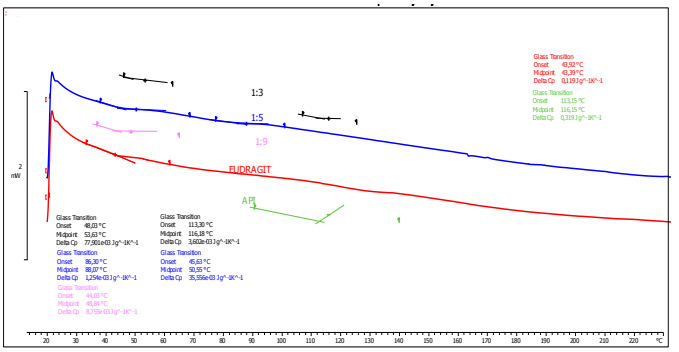
Mixing with API (DSC)



POLYMERS AS EXCIPIENTS

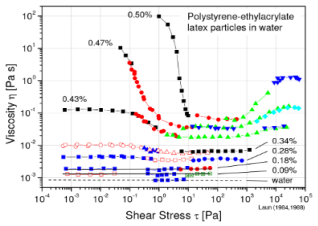
Solid dispersions: hot melt extrusion

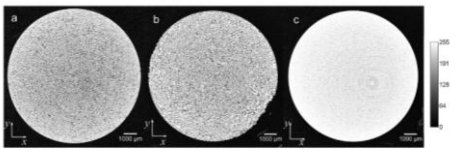
Mixing with API (DSC)

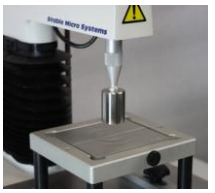


POLYMERS AS EXCIPIENTS – Modified release

Matrix formation for sustained release applications
- viscoelastic properties (DMA, rheology)
- water penetration monitoring (MRI)
- swelling and elasticity of tablets (TA)







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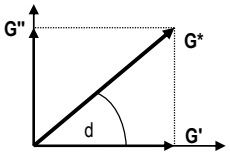
POLYMERS AS EXCIPIENTS – Viscoelastic properties

G'… Elastic modulus, storage modulus: *in phase with shear deformation*
G"… *Viscous modulus, loss modulus, out of phase with shear deformation*

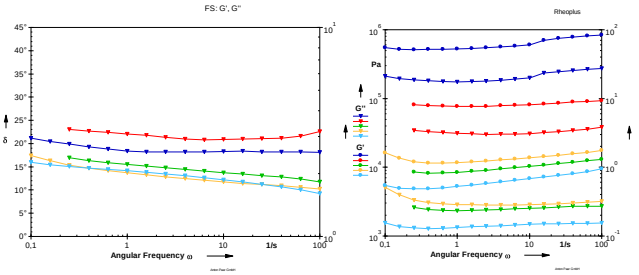
Phase shift(d) $\tan\delta = \frac{G''}{G'}$

Shear modulus G^* $|G^*| = \tau_a / \gamma_a$

Shear tension (τ_a); shear deformation(γ_a)



$|G^*| = \sqrt{|G'^2| + |G''^2|}$

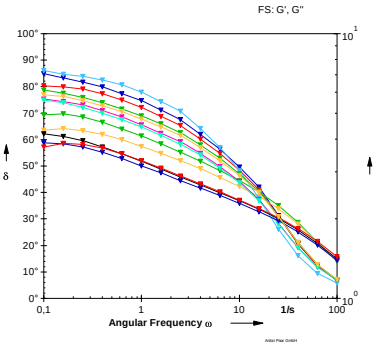


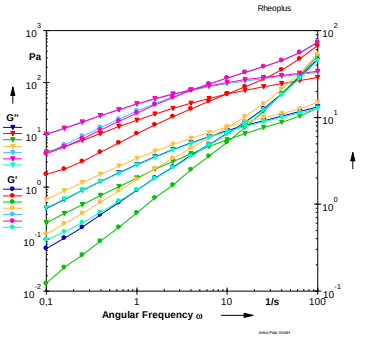
Gel-like liquid: G´ >> G´´
G´ in G´´ are not oscillation frequency dependent and they do not cross.

KRKA

POLYMERS AS EXCIPIENTS – Viscoelastic properties

Non-gelling´liquid: G ´´ >> G´
G´ in G´´ are oscillation frequency dependent and they do cross.



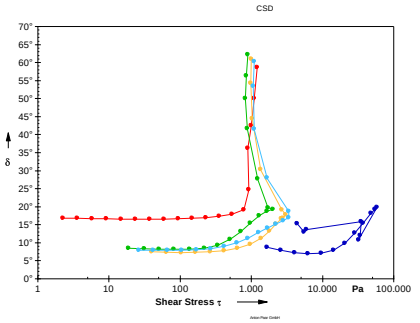


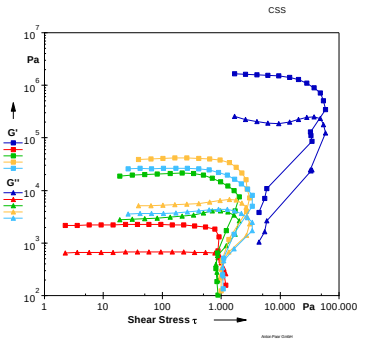
POLYMERS AS EXCIPIENTS – Viscoelastic properties

Oscillation tests

G ´, G ´´ and tanδ vs. Shear stress amplitude

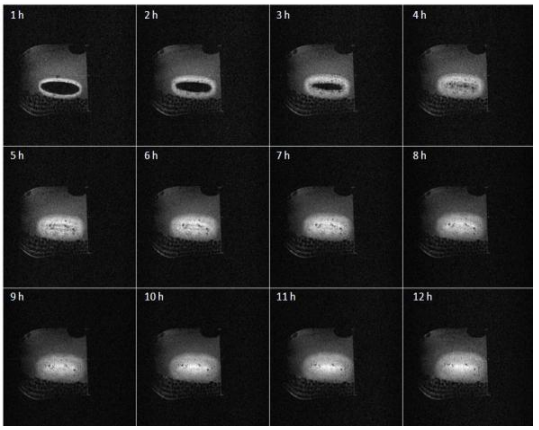
Gel structure break





POLYMERS AS EXCIPIENTS – MRI

Water penetration monitoring
Swelling



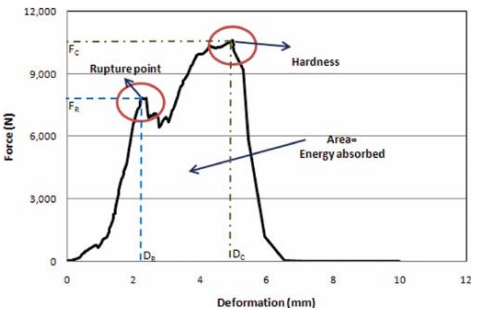
POLYMERS AS EXCIPIENTS – Texture analysis

MODES:

Compression

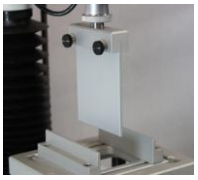
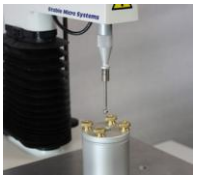
Deflection/Tension

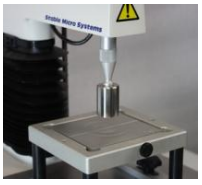
Adhesion



POLYMERS -texture analyser

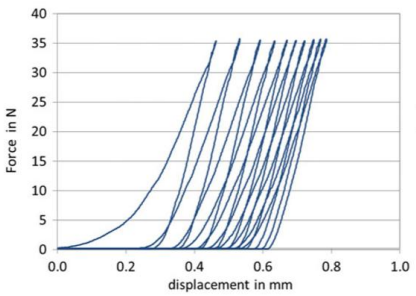
- three point bend rig
- film extensibility rig
- lid peel rig
- cylinder probes (compression)
- spherical probes (deflection)
- blister packs – force to burst

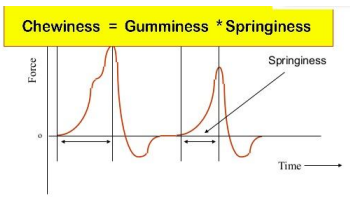






POLYMERS AS EXCIPIENTS – Chewable tablets

Hardness
Toughness, Firmness, Stiffness, Adhesion

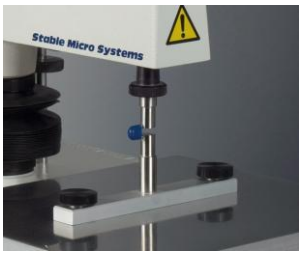




CAPSULES

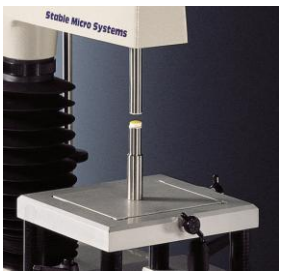
HPMC capsules

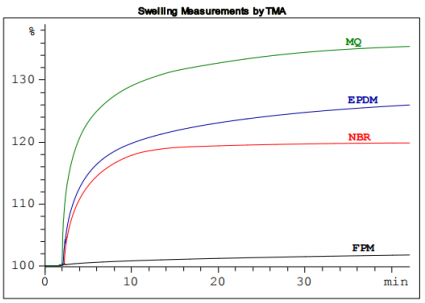
Gelatine capsules:
- Brittleness (water content, gel strength)
- Gel strength – Bloom value – texture analyser)
- Prelock Force (Texture analyser)
- Opening force (Texture analyser)



FILM COATING MATERIALS

- Key properties: solubility, viscosity, permeability, mechanical properties (viscoelastic properties: tensile strength, modulus of elasticity
Tensile strength: the maximum stress applied at the point at which the film breaks.
- AFM, SEM
- curing of the film – film strength
- attaching force (TA)
- swelling of Eudragit (TMA)





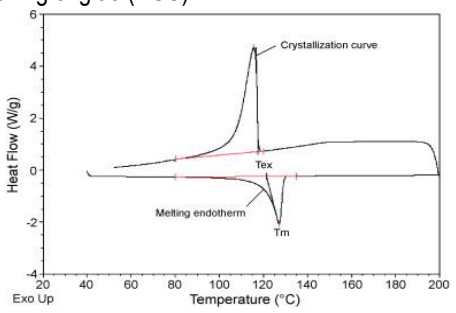
POLYMERS -texture analyser

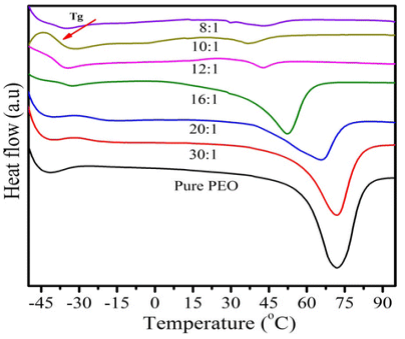
Fassihi Tablet Film & Coating Adhesion

A novel test method is demonstrated that uses a small metal plate that is placed on a tablet and then coated with a polymer film to measure the adhesion of the coating to the tablet. Clear difference are seen between three products with good repeatability. This method will allow formulators to explore the functionality of tablet coatings.

PACKAGING

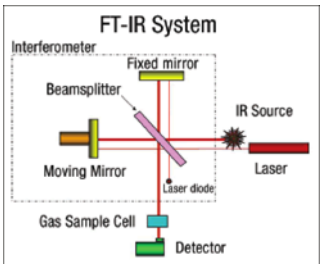
- identification (DSC)
- Purity (DSC)
- softening of glue (DSC)

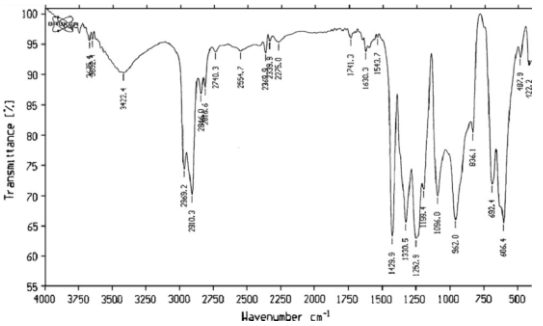




PACKAGING

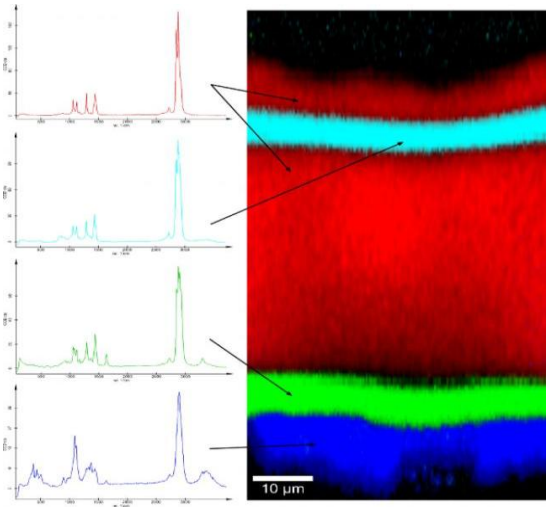
- identification (IR)





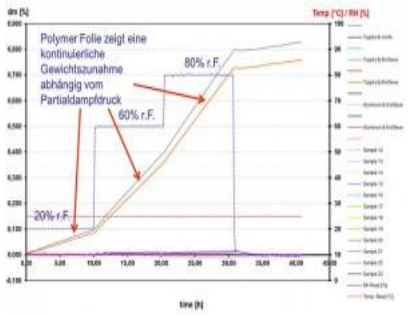
PACKAGING – microspectroscopic techniques

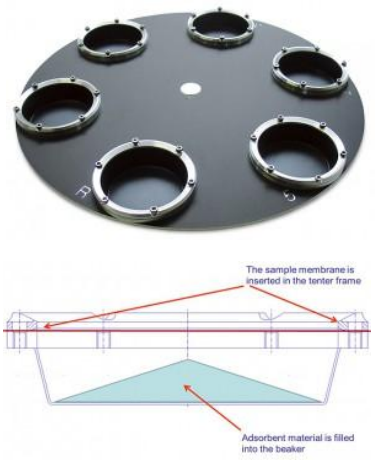
- thickness of layers (Raman, IR microspectroscopy)



PACKAGING

- permeability (SPS)





ACKNOWLEDGEMENTS

- Analytical Research and Development
  Krka, d.d., Novo mesto
- Structure Research Department, Krka, d.d., Novo mesto
- Management Board, Krka, d.d., Novo mesto
- SFD, Pharmaceutical Technology Section
- Natalija Zajc


KRKA
Living a healthy life.

Physicochemical, structural and biological properties of protein drugs

Assist. Prof. Tomaž Bratkovič, PhD

University of Ljubljana
Faculty of Pharmacy

14 June 2018, PIC Lek, Ljubljana
Polymers as Pharmaceutical Excipients and Active Ingredients

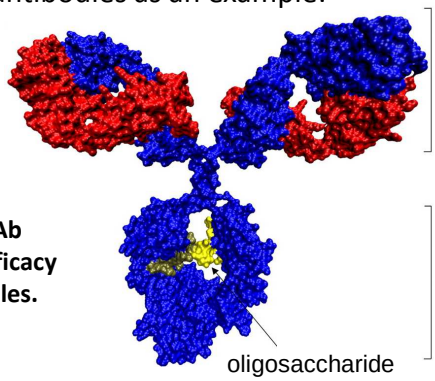
Complexity of protein drugs

small synthetic drug	**small protein drug**	**large (glyco)protein drug**
		
acetylsalicylic acid (21 atoms, 180 Da)	somatropin (3091 atoms, 22˙1 kDa)	antibody (IgG) (~25000 atoms, ~150 kDa)

Complexity of protein drugs

- **structural complexity** (size, fold, disulfide bonds, multiple domains/subunits, diversity of sugars…)
- **functional complexity** (multiple mechanisms of action)
- monoclonal antibodies as an example:

heavy chain
light chain
glycan



determines affinity and selectivity of antigen binding

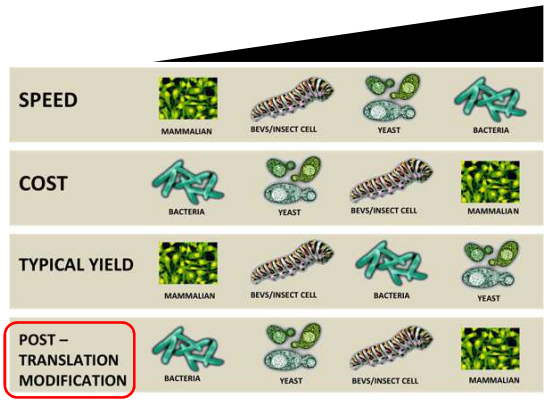
All parts of mAb determine the efficacy and safety profiles.

determines effector functions (mechanisms of action) and $t_{1/2}$

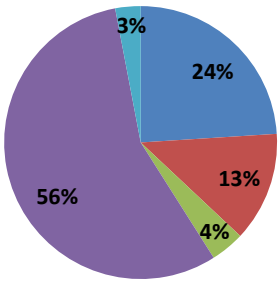
Manufacturing biologicals

- *„The process is the product.“*

A. Choosing the expression system



https://aibn.uq.edu.au/protein-expression

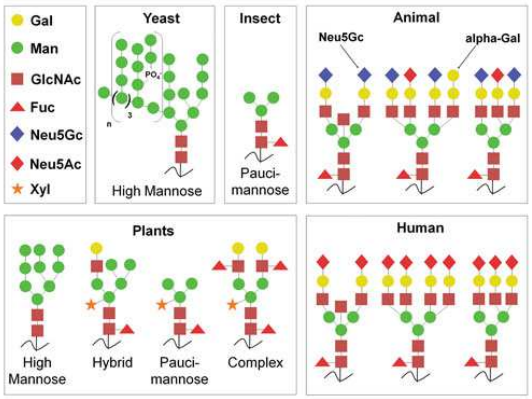


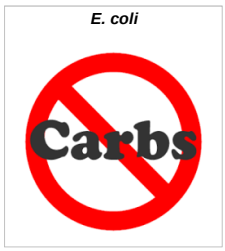
E. coli
yeast
insect cells
mammalian cells
transgene plants and animals

Manufacturing biologicals

- *„The process is the product."*

A. Choosing the expression system - glycosylation

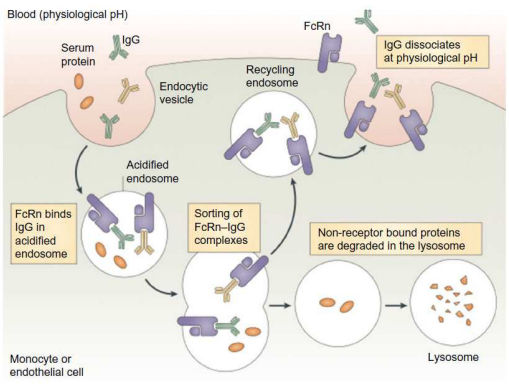




De Palma A. (2013) *Genetic Engineering and Biotechnology News* 33(1).

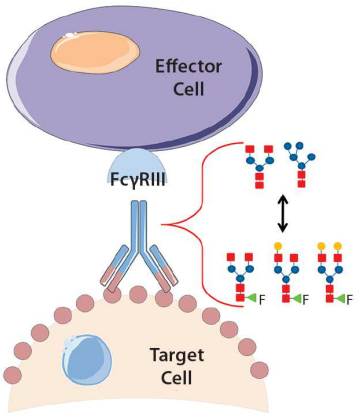
Effect of glycosylation on PK and PD

PK



Roopenian DC & Akilesh S (2007) *Nat Rev Immunol* 7: 715-725.

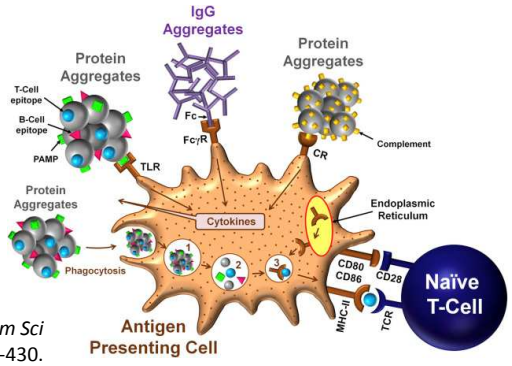
PD



Ho TD *et al. BioProcess International*, April 2016.

Effect of glycosylation on immunogenicity

- foreign sugars as **neoantigens**
- **lack of glycosylation** can **expose cryptic epitopes**
- glycosylation **augments solubility** and **supports proper protein folding** → aggregation of unglycosylated proteins

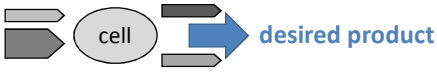


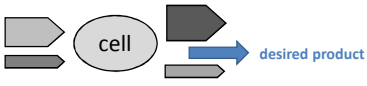
Moussa EM *et al*. (2016) *J Pharm Sci* 105(2): 417-430.

Manufacturing biologicals

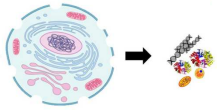
- *„The process is the product."*

B. Bioreactor conditions

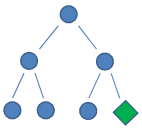




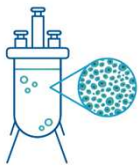
metabolism



cell lysis and release of proteases/glycosidases



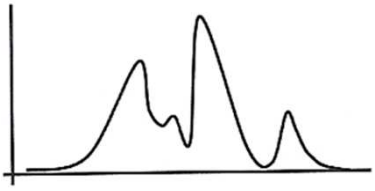
mutations

Milky way	120 hL bioreactor
	
~10^{11} stars	~10^{14} cells

Manufacturing biologicals

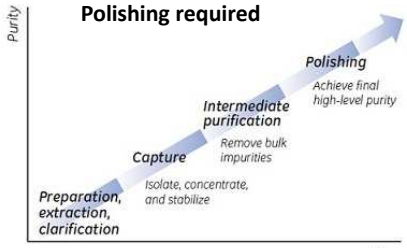
- *„The process is the product.“*

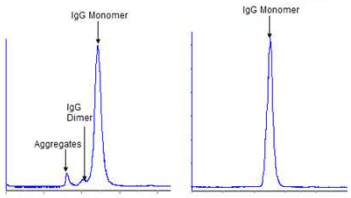
C. Isolation and **purification**



fuzzy separation – co-purification of impurities
(host cell proteins, breakdown products, aggregates, glycosylation isoforms…)

Polishing required



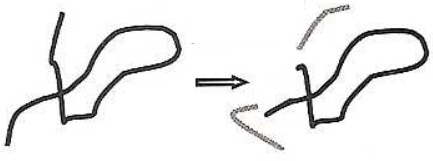


Manufacturing biologicals

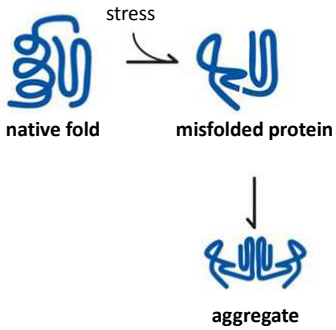
- *„The process is the product.“*

D. Formulation and **storage**

temperature, pH, oxygen,
light, proteases,
glycosylases, metal ions…

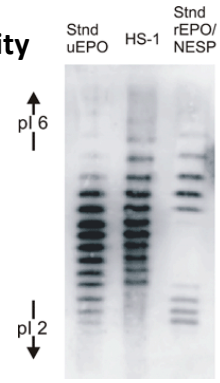


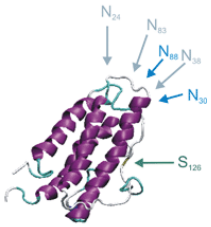
hydrolysis, oxidation, deamidation, denaturation & aggregation…



Features of protein drugs

- **(micro)heterogeneity**





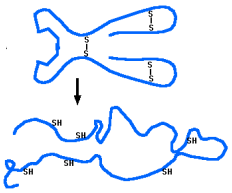
Defaus S *et al*. (2014) *Analyst* 139: 2944–2967.

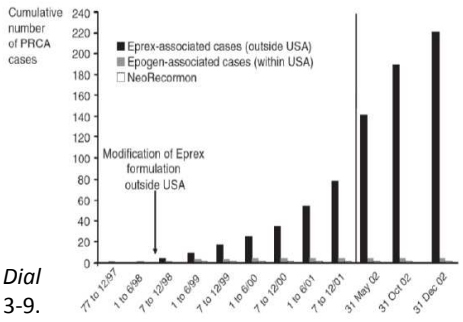
- **batch-to-batch variability**

- **complex analytics**

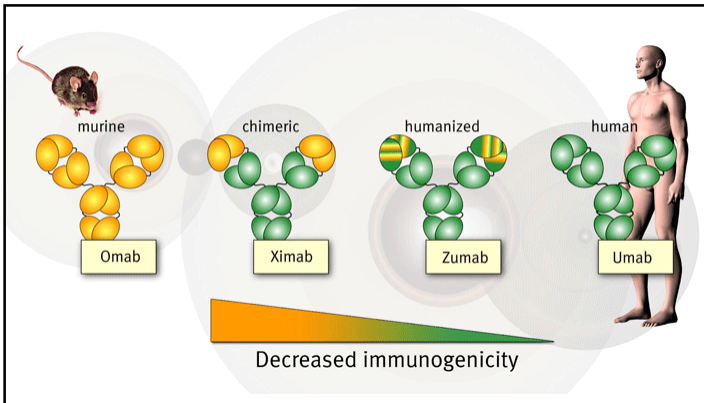
Features of protein drugs

- **high structural complexity → specificity**
- **instability**
- **parenteral application required**
- **high development and <u>production</u> costs → high price**
- **immunogenicity:**
  - **↓ $t_{½}$ and/or**
  - **loss of efficacy**



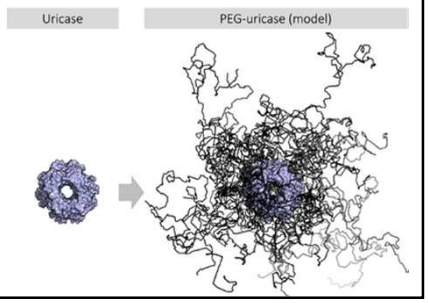


Shellekens H (2005) *Nephrol Dial Transplant* 20: 3-9.

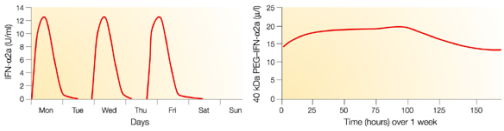


Humanization of antibodies

PEGylation



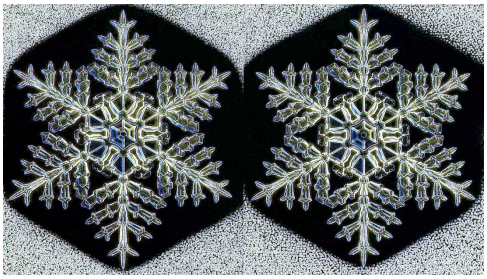
Limiting immunogenicity of biologics



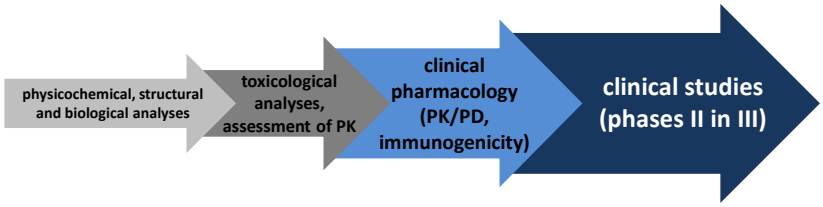
Harris JM & Chess RB. Nat Rev Drug Disc 2: 214-221.

Biosimilars

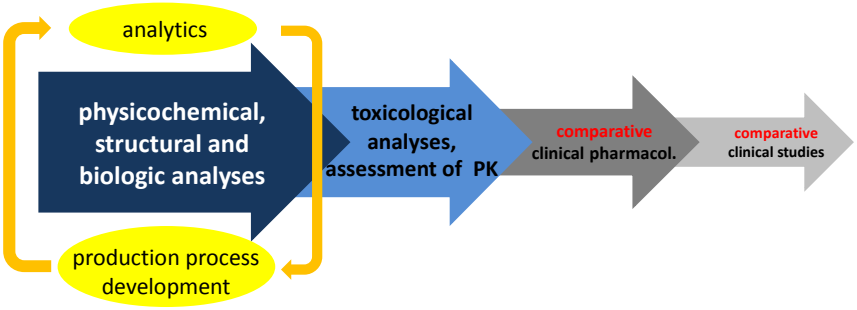
- concept analogous to generic drugs
- (im)perfect copy of the reference (originator) drug
- ***comparability***, ***similarity*** and ***extrapolation*** concepts
- *know-how*: - consistency of production process
  - top-notch analytics



Development of innovative drugs

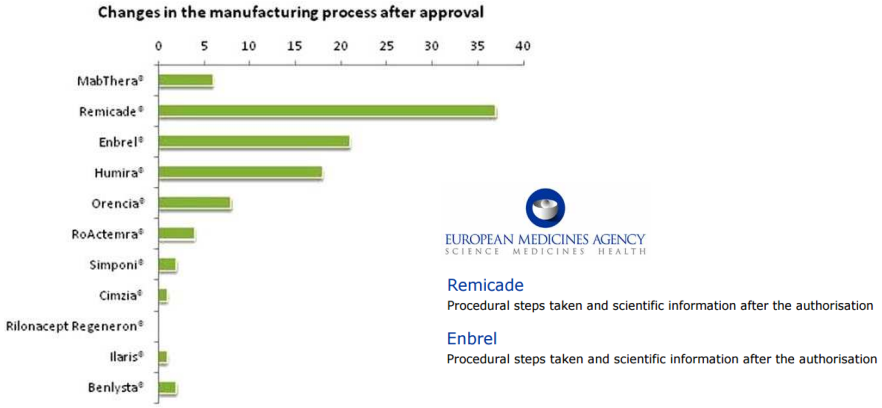


Development of biosimilars



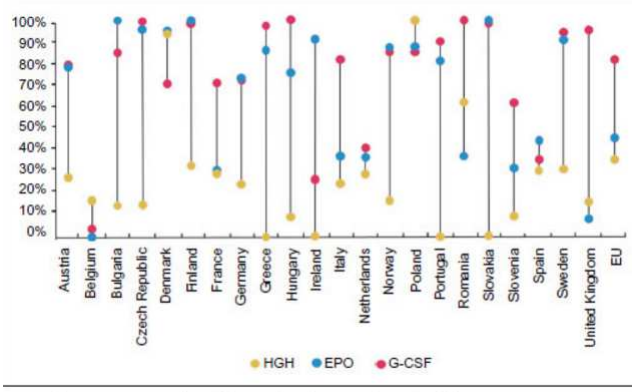
adapted from Olech E (2016) *Semin Arthritis Rheum* 45: S1-S10.

Changes of reference biologics during their life cycle



Schneider CK (2013) *Ann Rheum Dis* 72(3): 315-318.

Penetration of biosimilars in the EU



EPO: erythropoietin; G-CSF: granulocyte colony-stimulating factor; HGH: human growth hormone.
Source: IMS Health Midas 2015

Ekman *et al.* (2016), *GaBI Journal* 5(2): 84-88.

Biosimilars in the EU

Medicine	API	Therapeutic indications	Date of MA
Abasaglar (Abasria)	insulin glargine	diabetes	09/09/2014
Abseamed	epoetin alfa	anemia (cancer, chronic kidney failure)	28/08/2007
Accofil	filgrastim	neutropenia	18/09/2014
Bemfola	follitropin alfa	anovulation	27/03/2014
Benepali	etanercept	PA, RA, psoriasis	14/01/2016
Binocrit	epoetin alfa	anemia (chronic kidney failure)	28/08/2007
Biograstim	filgrastim	cancer, hematopoietic stem cell transplantation, neutropenia	15/09/2008
Epoetin Alfa Hexal	epoetin alfa	anemia (cancer, chronic kidney failure)	28/08/2007
Filgrastim Hexal	filgrastim	cancer, hematopoietic stem cell transplantation, neutropenia	06/02/2009
Filgrastim ratiopharm	filgrastim	withdrawn	15/09/2008
Flixabi	infliximab	PA, RA, UC, CD, psoriasis, AS	26/05/2016
Grastofil	filgrastim	neutropenia	18/10/2013
Inflectra	infliximab	PA, RA, UC, CD, psoriasis, AS	10/09/2013
Inhixa	enoxaparine	venous thrombembolism	15/09/2016
Lusduna	insulin glargine	diabetes	04/01/2017
Movymia	teriparatide	osteoporosis	11/01/2017
Nivestim	filgrastim	cancer, hematopoietic stem cell transplantation, neutropenia	08/06/2010
Omnitrope	somatropin	growth disorders (hGH deficit), Prader-Willi syndrome, Turner syndrome	12/04/2006
Ovaleap	follitropin alfa	anovulation	27/09/2013
Ratiograstim	filgrastim	cancer, hematopoietic stem cell transplantation, neutropenia	15/09/2008
Remsima	infliximab	PA, RA, UC, CD, psoriasis, AS	10/09/2013
Retacrit	epoetin zeta	anemia (autologous blood transfusion, cancer, chronic kidney failure)	18/12/2007
Silapo	epoetin zeta	anemia (autologous blood transfusion, cancer, chronic kidney failure)	18/12/2007
Terrosa	teriparatide	osteoporosis	04/01/2017
Tevagrastim	filgrastim	cancer, hematopoietic stem cell transplantation, neutropenia	15/09/2008
Thorinane	enoxaparin	venous thrombembolism	15/09/2016
Truxima	rituximab	RA, chronic B-cell leukemia, non-Hodgkin limphoma, microscopic polyangiitis, Wagner granulomatosis	17/02/2017
Valtropin	somatropin	withdrawn	24/06/2016
Zarzio	filgrastim	cancer, hematopoietic stem cell transplantation, neutropenia	06/02/2009

Biosimilars in the EU

Approved in the last year alone:

Amgevita
(adalimumab)

Blitzima
(rituximab)

Cyltezo
(adalimumab)

Erelzi
(etanercept)

Imraldi
(adalimumab)

Insulin lispro Sanofi
(insulin lispro)

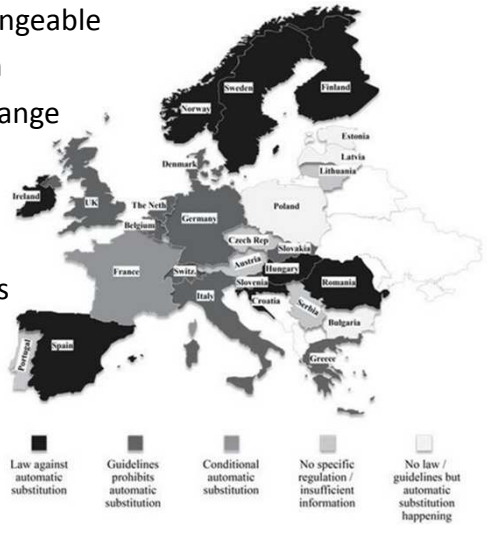
Mvasi
(bevacizumab)

Ontruzant
(trastuzumab)

Solymbic
(adalimumab)

Interchangeability?

- **not** automatically interchangeable
- only by specialist physician
- patient informed of the change
- patient closely monitored
- physician-pharmacist communication essential
- pharmacovigilance systems (patient registries)



GDD

Breast Cancer and Stromal Cell Co-cultures Models for Immunoconjugate Therapy Optimisation

Barbara Podobnik
June 14, 2018

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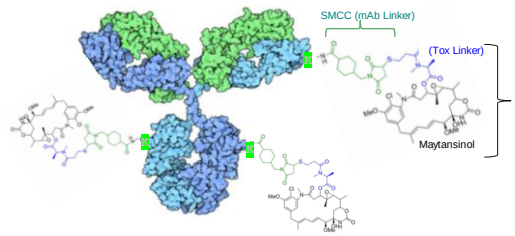
Antibody Drug Conjugates
Paul Ehrlich – „Magic Bullet" Concept

Paul Ehrlich (1854 – 1915) - a physician and scientist

- Ehrlich reasoned that if a compound could be made that **selectively** targeted a disease-causing organism, then **a toxin** for that organism could be **delivered** along with the agent of selectivity.
- The antibody drug conjugates combine biologic and cytotoxic mechanism into one targeted therapy
- Characteristic structure of ADC:

Antibody, linker-conjugate and toxin!



DM1, derivative of maytansine:

- **Mitotic arrest**
- **Apoptosis**
- **Mitotic catastrophe**
- **Disrupted intracellular trafficking**

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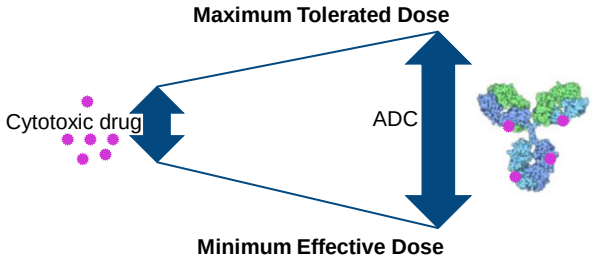
ADCs - Antibody Drug Conjugates

New generation of anticancer drugs.

- Combines **high potency** of toxins and high **specificity** of monoclonal antibodies (mAB), providing...
- **Increased therapeutic window** by targeted delivery and reduced systemic toxicity.



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Breast Cancer (BCa) types and therapeutic based classification

Biological targeting based BCa therapy is aimed to inactivate two types of receptors:

- **Estrogen receptors (ER) and Progesterone receptors (PR)**
- **HER2/neu receptor**

Metastatic BCa clasification

Hormone ER receptor negative

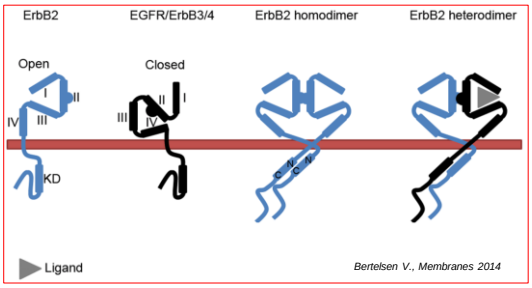
Hormone PR receptor negative

HER2 negative

HER2 positive

Triple-positive

Triple-negative



Her2/ErbB2 receptor is amplified and overexpressed in 15-20% of all breast cancers, where it is associated with more aggressive disease and poor prognosis.

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Treatment Landscape in ~~HER2+~~ Metastatic BCa

The total **incidence of breast cancer ww is expected to increase by 19.6%** over the forecast period from 2014 to 2034, resulting in **641.410 newly diagnosed cases.**

	1st Line	2nd Line	3rd, 4th Line or greater
Number of Patients (per year in US, EU and Japan)	**21.500**	**16.500**	**15.400**
Standard of Care Therapy	Pertuzumab + **Trastuzumab +** taxane	**Kadcyla®**	No clear SOC
Median PFS	**18 months [1]**	**10 months [2]**	**3 month [3]**
Clinical outcome	~ 30% progress in 12 months **~ 20% do not respond**	~ 30% progress in <6 months **~ 55% do not respond**	Rapid progression **~ 90% do not respond**

Urgent need for improvement of therapies in late line MBCa.

(1) CLEOPATRA, Baselga, et al., 2012 (2) EMELIA, Verma, et al., 2012 (3) TH3RESA, Krop et al., 2014

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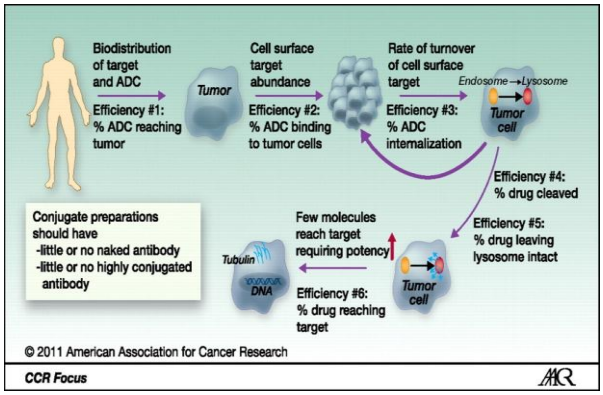
Insight look into ADC's Mode of Action



Teicher B A , and Chari R V Clin Cancer Res 2011;17:6389-6397

1. Biodistribution
2. Internalization
3. Lysosome & endosme merging - acidification
4. Proteolytic cleavage in late endosomes

↓

5. Pay-load release

↓

6(a) Microtubule disassembly

6(b) DNA minor grove binding

↓

Toxic effect!

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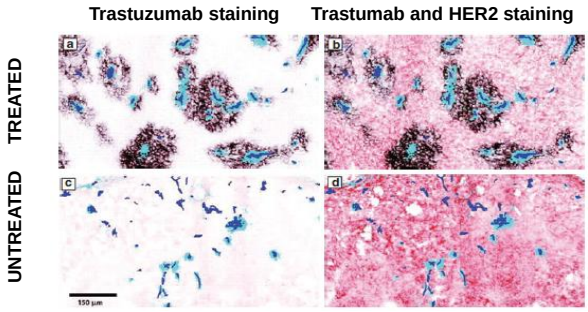
Immunoconjugates Against Solid Tumors: Mind the Gap!

Obstacles to achieve efficacy with mAb therapy

- **Impaired mAb distribution**
- **Limited delivery to tumor sites**
- Insufficient trafficking of effector cells to tumor
- Antigenic heterogeneity (intratumoral and intertumoral)
- Shedding and internalization of target antigens
- Insufficient tumor specificity of target antigens



Heterogeneous extravascular distribution of trastuzumab in high HER2 expressing mice xenografts

Baker, J.H. *et al.*, *Clin. Cancer Res.* 14, 2171–2179 (2008)

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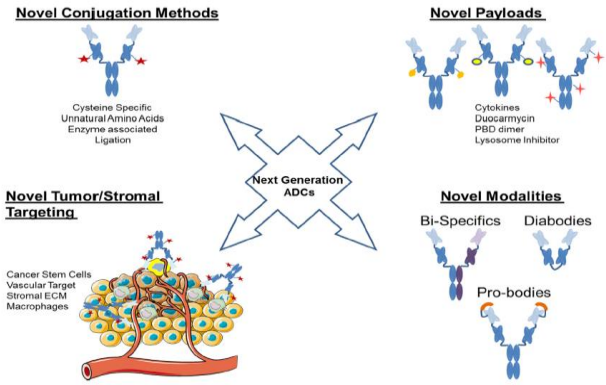
Opportunities to Improve ADCs



- **Site specific conjugation** chemistries
- **Advanced ADC** technologies related to linkers and payload for enhanced safety& efficacy
- Use of **antibody fragments** instead of full mAbs for future ADCs carrier

Relevant tumour models needed!

Seminars in Oncology, Vol 41,2014, 637, Pfizer Inc

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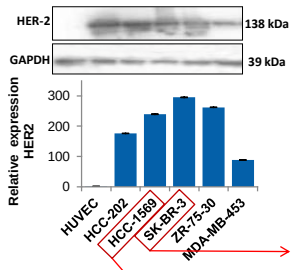
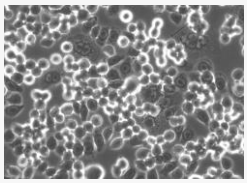
1st AIM: To Identify Suitable MBCa Cells Lines for *in vitro* Testing

HER2 is differentially expressed in BCa cell lines



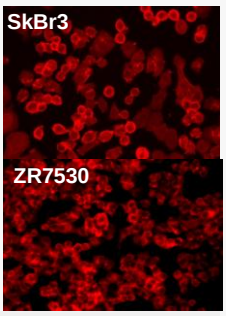
Cellular morphology - variabity depends on BCa tissue origin

SkBr3

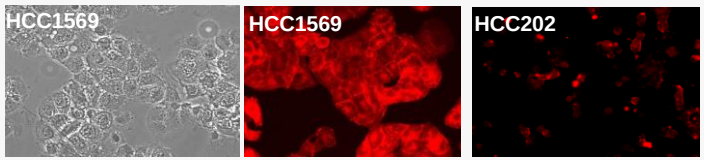


Semi-adherent cell lines from pleural effusion

anti HER2 staining



Adherent primary cultures from solid tumors



anti HER2 staining

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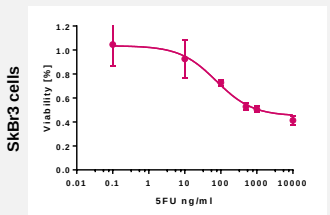
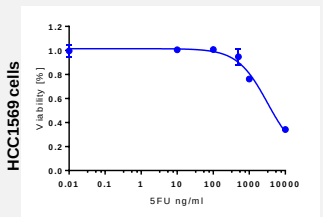
2D Monolayes: BCa Cell Lines Exhibit Distinct Drug Response

5'FU treatment (4 days)

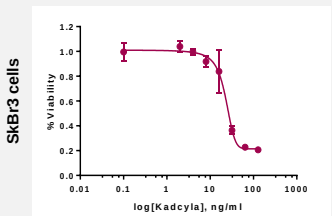


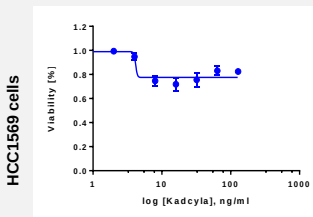


SkBr 3 cells (*pleural effusion*) **and HCC1569 cells** (*primary tumour tissue origin*) **exhibit different sensitivity to 5'FU** (*antimetabolite – pyrimidine analogue causing DNA damage*) **and Kadcyla**.

HCC1569 cells are significantly more resistant to Kadcyla and slightly more to 5'FU than SkBr3 cells.

Kadcyla treatment (4 days)





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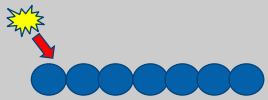
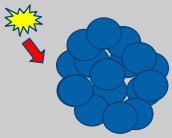
2nd AIM: To Establish 3D Cell Culture Models



All cells equally affected



Cells within the spheroid are differentially affected due to limited penetration, cell shielding effect and differences in microcirculation

2D monolayer cell models vs. 3D spheroid models

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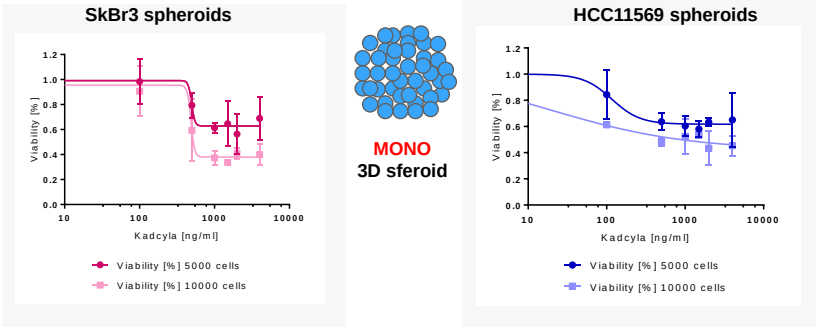
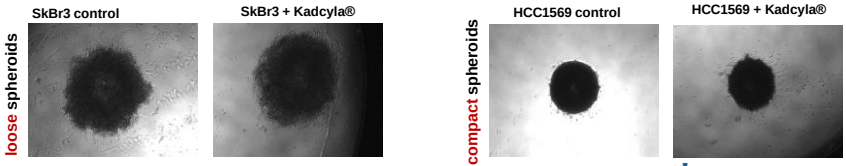
Belated Cell Death Response in 3D Monospheroids Compared to Monolayers



Metabolic activity and the level of spheroid compaction dictates the SkBr3 and HCC1569 response to Kadcyla®.



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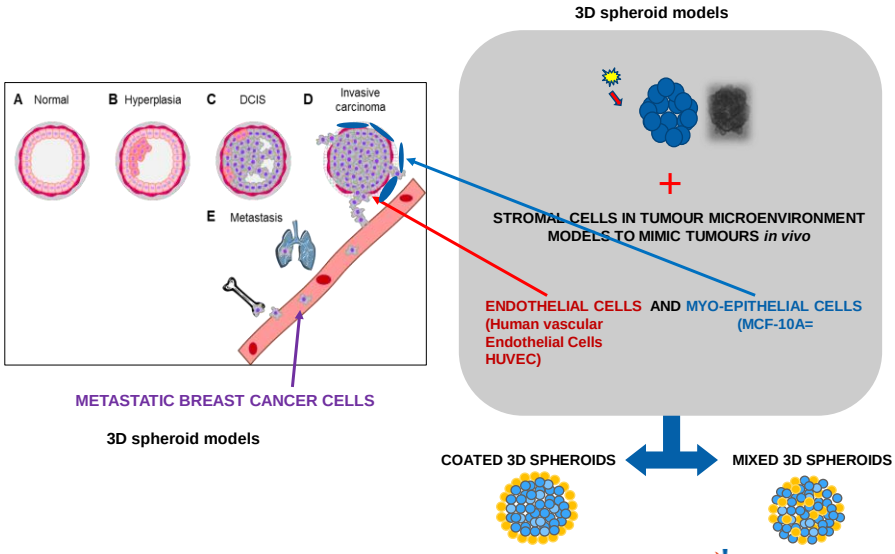
3rd AIM: 3D BCa Models in Co-cultures with Stromal Cells To Mimick Tumour Microenvironment



Technical Research and Development

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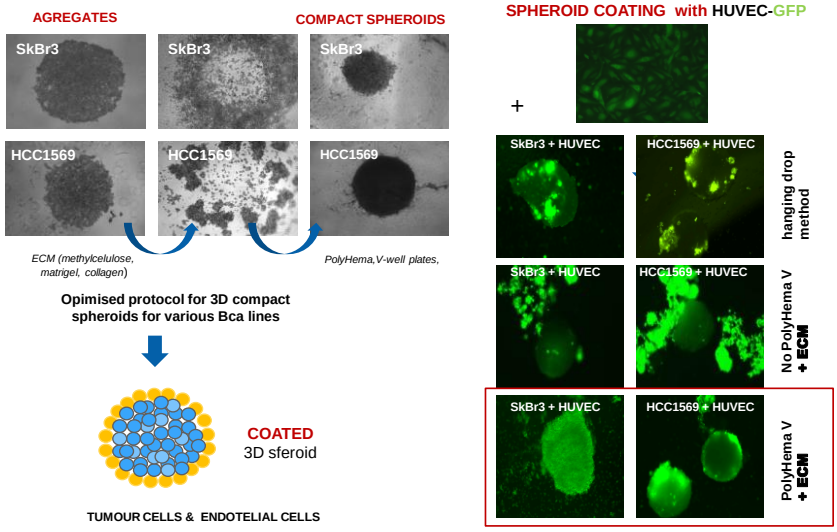
The Establishment of Compact COATED Spheroids of BCa Cells Coated with HUVECs



Technical Research and Development

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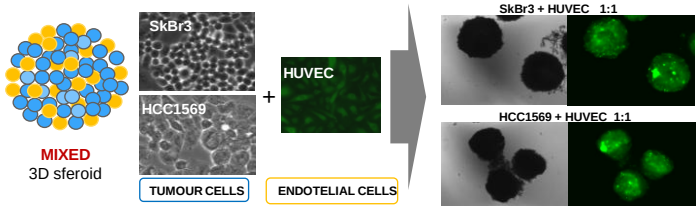
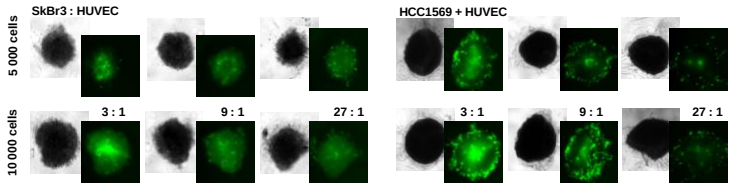
The Establishement of Compact MIXED Spheroids of BCa Cells with HUVEC

- **Better compaction in directly mixed spheroids compared to mono- and coated co-cultured spheroids.**



- **Observation: Endothelial cells overgrowth in mixed spheroids**
- **Optimisation: BCa /HUVEC cells' ratios & total cells number (spheroid size)**



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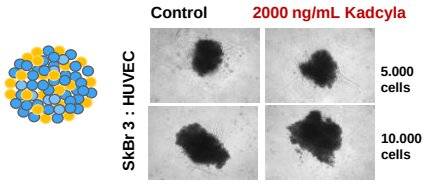
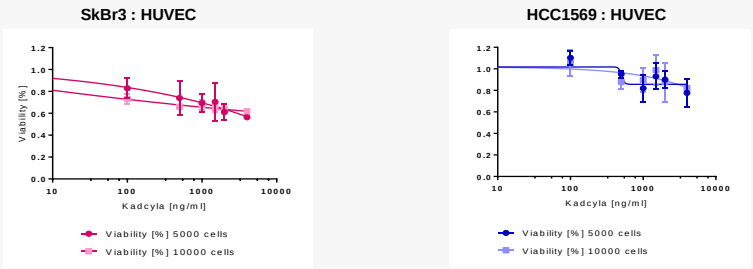
Kadcyla® 's Response in 3D BCa / HUVEC co-cultured spheroids

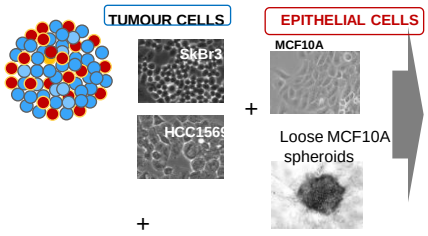


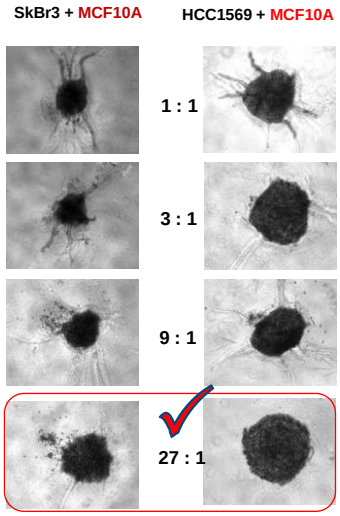
- **Fast overgrowth of HUVEC cells reduces the sensitivity and consistency of the model.**
- Hypoxia in the spheroid may induced HIF1a promoting vascular endothelial factor (VEGF) expression, resulting in accelerated HUVEC proliferation



NOVARTIS

The Establishment of Compact MIXED Spheroids of BCa Cells Coated with Myo-epithelial cells

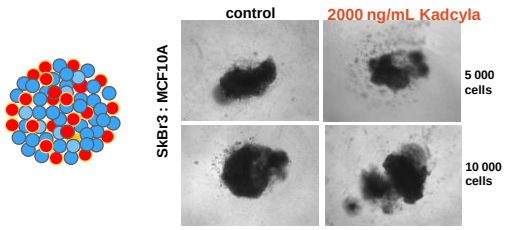


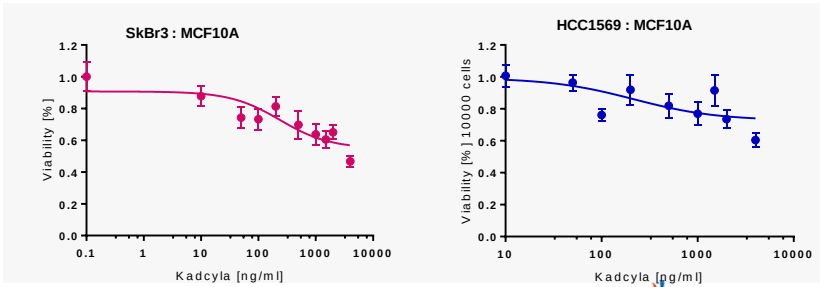


- At lower cell to cell ratios the epithelial MCF10A cells grow over the spheroids and form compact outgrowths as buds from the outer layer.
- 27 to 1 cell to cell ratio was selected for further testing.

NOVARTIS

Kadcyla® 's Response in 3D BCa / Myoepithelial MCF-10A Co-cultured Spheroids

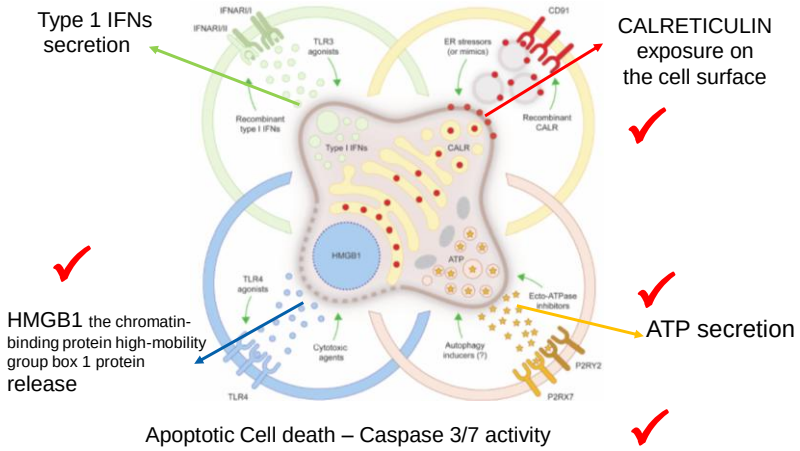




NOVARTIS

4th Aim: Is Kadcyla® Inducing Immunogenic Cell Death?

Immunogenic Cell Death (ICD) biomarkers:



Bozu et all, Frontieres in Immunology 2015

NOVARTIS

Experimental set up for ICD Evaluation

Kadcyla®, 5'-FU

Spheroid formation
Cancer cells:MCF10A = 27:1

Treatment period after drug administration

Day 1 Day 2 Day 3

Day 1 Day 2 Day 3 Day 4 Day 5 Day 6

CONTROL - BASAL
- Caspase 3/7 activity
- ATP release

- ✓ **Caspase 3/7 activity** assay (media)
- ✓ **ATP –release** assay (media)
- ✓ Metabolic activity assay
- ✓ **CalR** and **HMGB1** translocation detection by flow cytometry

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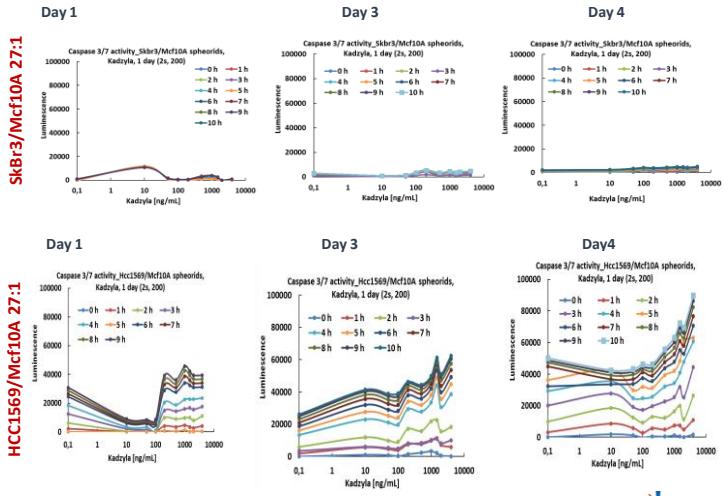
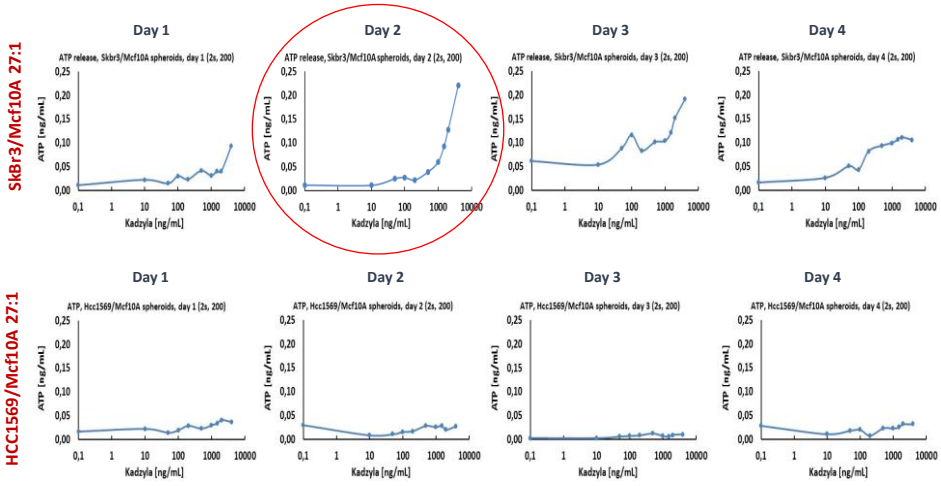
Kadcyla® induces Caspase 3/7 Activity in HCC1569/MCF10A Mixed Spheroids 10 times more than in SkBr3/MCF10A



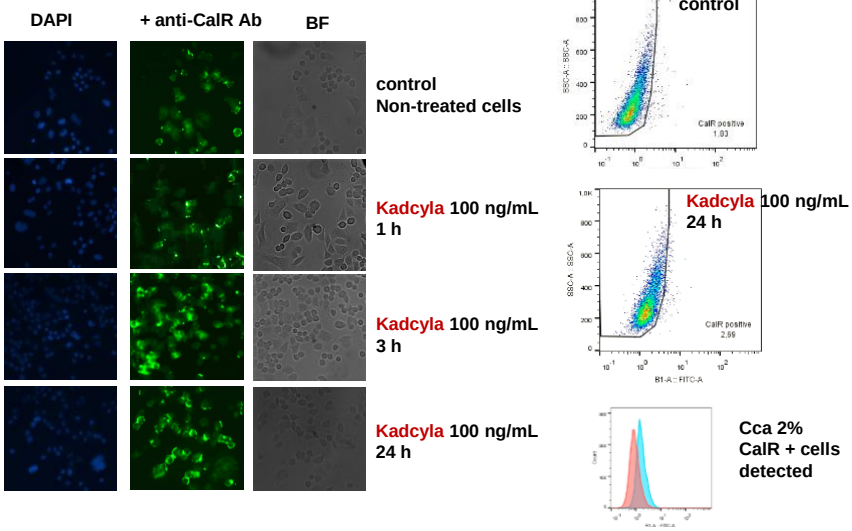
NOVARTIS

Kadcyla® induces Basal ATP release in SkBr3/MCF10A Mixed Spheroids but not HCC/MCF10A Mixed Spheroids



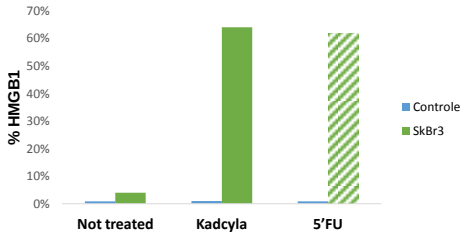
NOVARTIS

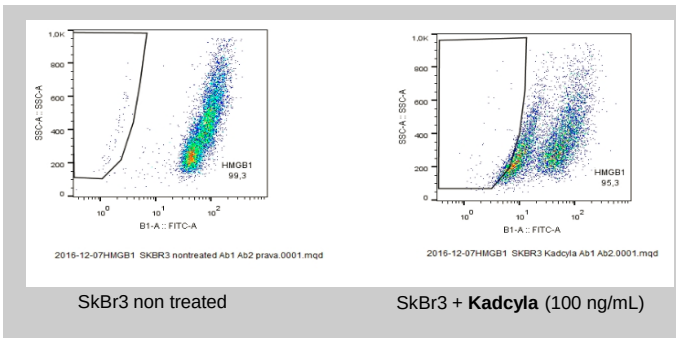
Kadcyla® induces Calreticulin ctranslocation on the SkBr3 cell surface



NOVARTIS

Kadcyla® induces HMGB1 release in SkBr3 cells





SkBr3 non treated

SkBr3 + **Kadcyla** (100 ng/mL)

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In conclusion:

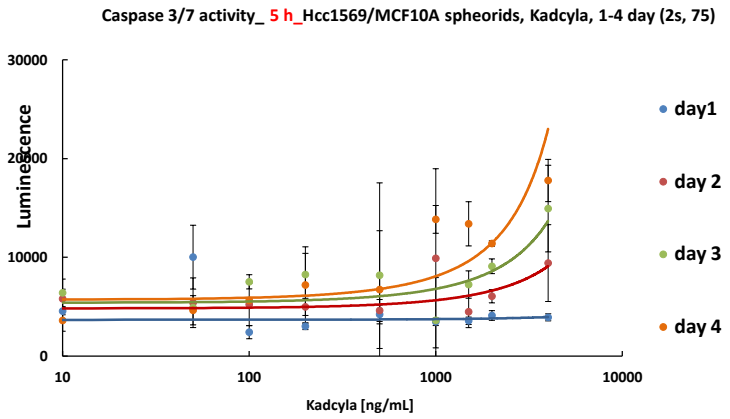
- Different resistance to cytotoxic drugs observed between the treated types of mono and mixed spheroids indicate that the stromal part of the tumor microenvironment may play a crucial role in tumor resistance to ADCs, such as Kadcyla®.
- Established *in vitro* spheroid models will serve as a valid tools enabling optimized design of next generation aHer2 ADCs.

NOVARTIS

Thank You

NOVARTIS

Kadcyla® induces Basal ATP release in SkBr3/MCF10A Mixed Spheroids but not HCC/MCF10A Mixed Spheroids



NOVARTIS

Nanosized protein complexes in biology and their application in biotechnology

Assist. Prof. Marjetka Podobnik, PhD
Department of Molecular Biology and Nanobiotechnology
National Institute of Chemistry, Ljubljana, Slovenia

29th Symposium of a Section of Pharmaceutical Technologists
PIC Lek, June 14th, 2018

National Institute of Chemistry, Ljubljana, Slovenia

since 1946

Life sciences and material sciences

- 12 Departments
- 309 Employees
- 140 Researchers with PhD
- 74 PhD students

- Expanding knowledge of chemistry and associated studies
- Transferring knowledge to younger generations
- Application of newly acquired knowledge to industry

Research at Department of Molecular Biology and Nanobiotechnology

Molecular interactions & mechanism of action

Pore-forming proteins

1. Mechanistic details of pore formation at the molecular level
2. Effect of membrane composition on protein complexes
3. Effects of proteins on membrane properties
4. Selective/specific binding of membrane lipids
5. Evolution of membrane binding sites

Other proteins involved in host-pathogen interactions

1. Structure and function
2. Mechanistic synergy with pore forming proteins

Filamentous plant viruses and virus-like particles

1. Structure
2. Assembly mechanism
3. Interactions with other virus and plant molecules

Application

Nanobiotechnology: sensing, drug delivery, drug design (pesticides/insecticides), vaccine development etc.

Department of Molecular Biology and Nanobiotechnology

Methodological approaches

Molecular biology and biochemistry: recombinant protein production (bacteria, yeast, insect cells), well-equipped for protein purification, protein and lipid biochemistry

Cell biology: flow cytometry, confocal microscopy, cell biology laboratory

Structural approaches: X-ray crystallography, atomic force microscopy (AFM), small angle X-ray scattering (SAXS), transmission electron microscopy (TEM), cryo-EM, NMR

Biophysics: surface plasmon resonance (SPR), microscale thermophoresis (MST), isothermal microcalorimetry (ITC), quartz crystal microbalance (QCM), planar lipid membranes systems, fluorescence spectroscopy, microscopies, circular dichroism, dynamic light scattering (DLS)

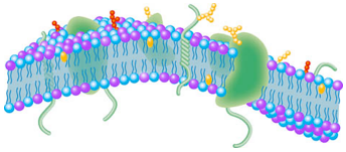
Others: Synthetic biology, ribosomal display, *in vitro* protein production.

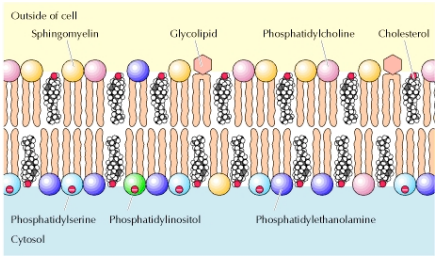
Infrastructural Centre for Molecular Interactions Analysis

http://www.molekulske-interakcije.si/en/index.html

Cellular membranes

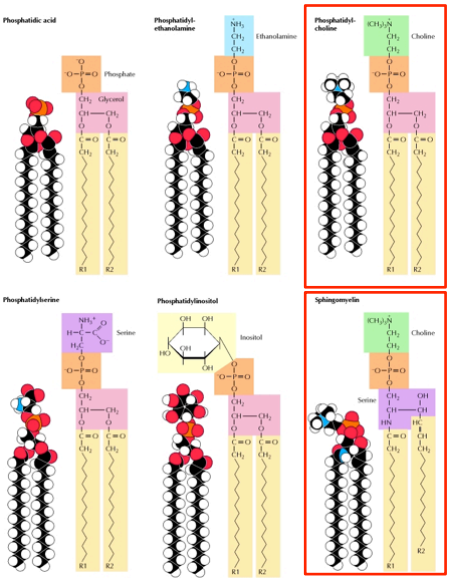
Biological membranes – vital & vulnerable



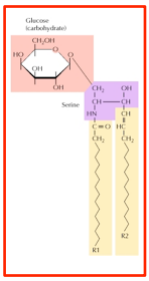


https://www.ncbi.nlm.nih.gov/books/NBK9898/

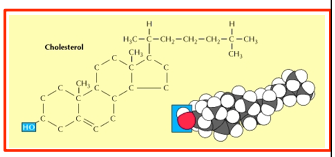
Membrane lipids



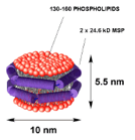
phospholipids



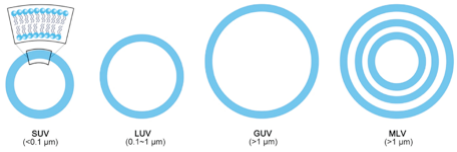
glycolipids



cholesterol



nanodiscs



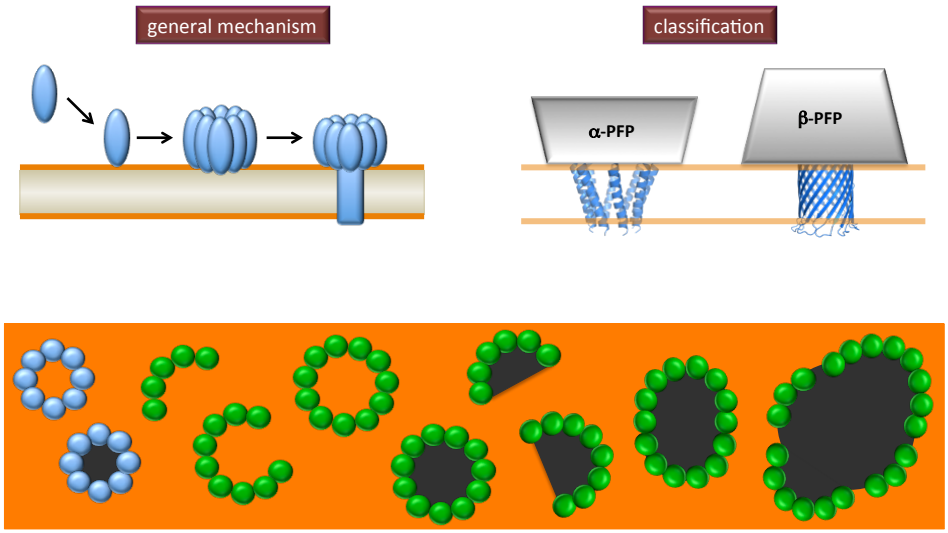
liposomes

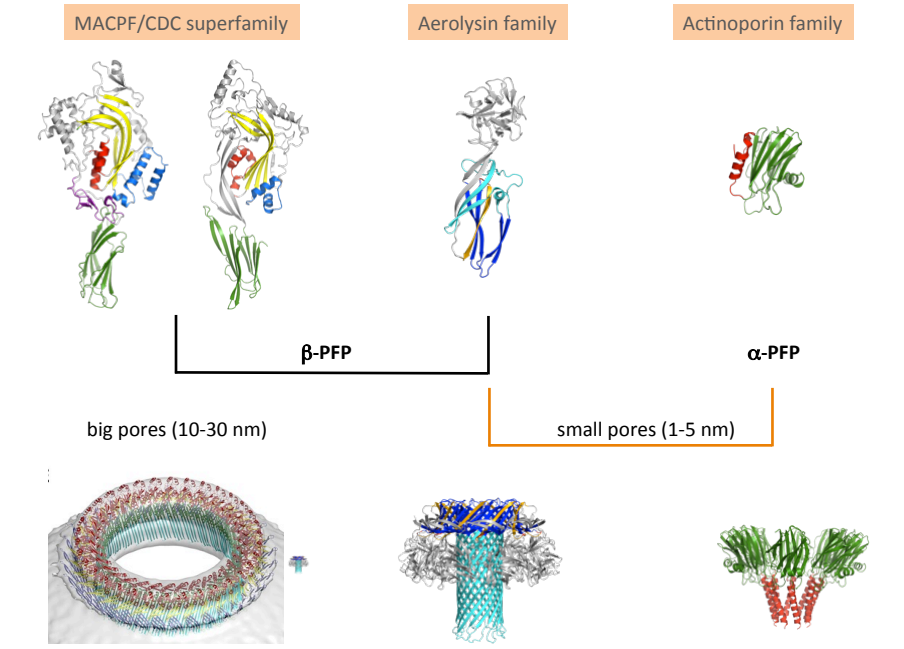
https://www.ncbi.nlm.nih.gov/books/NBK9898/

Li et al., Rev Analyt Chem 2017, DOI: https://doi.org/10.1515/revac-2017-0012

Pore Forming Proteins (PFPs)

Attack, defense, immune system, development, digestion

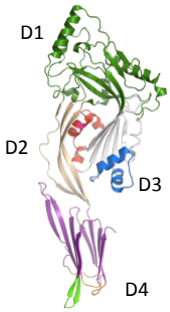




van Pee et al, eLife 2017;10.7554/eLife.23644 Podobnik et al. (2016) Nat Commun., 7:11598 Tanaka et al., Nat Commun. 2015;6:6337

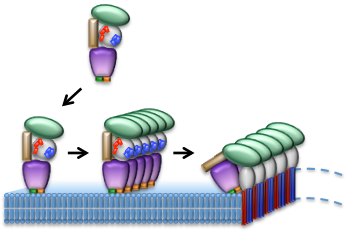
Pore formation by Cholesterol Dependent Cytolysins (CDCs)

Need high cholesterol content > 30 mol %

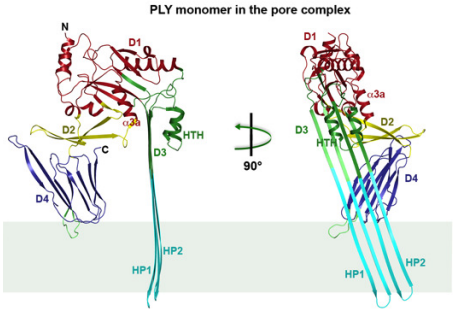


Listeriolysin O
(*Listeria monocytogenes*)

Köster et a.l, Nat Commun. 2014;5:3690



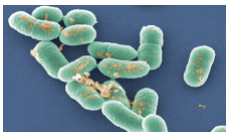
Podobnik et al., Sci Rep. 2015,5:9623



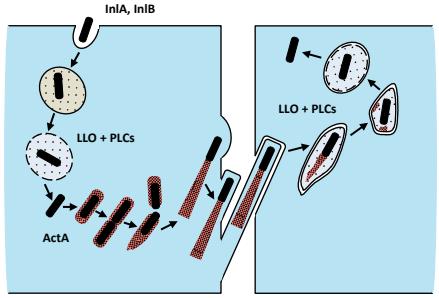
van Pee et al., eLife 2017;10.7554/eLife.23644

Listeriolysin O (LLO) – intracellular toxin

- Expressed by a Gram+ bacterium *Listeria monocytogenes*
- Causes *listeriosis* (immunocompromised people and animals!)
- Gastroenteritis, meningo-encephalitis, sepsis, abortions
- Treatment with antibiotics; 20 – 30 % mortality rate
- LLO as a Swiss army knife: primarily intracellular toxin, however, also acts extracellularly
- Higher stability at acidic pH than at neutral at temperatures above 30°C
- Unique plasticity of LLO pores

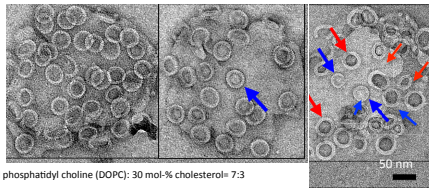


http://study.com/academy/lesson/listeria-monocytogenes-symptoms-treatment.html



Unique plasticity of LLO pores

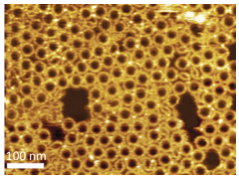
Pneumolysin *(Streptococcus pneumoniae)*



di-oleyl phosphatidyl choline (DOPC): 30 mol-% cholesterol= 7:3

van Pee et al, eLife 2017;10.7554/eLife.23644

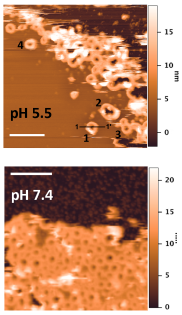
Perfringolysin *(Clostridium perfringes)*



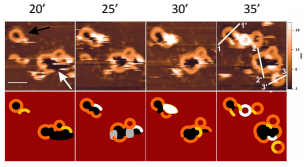
egg phosphatidylcholine:cholesterol=1:1

Czajkowsky et al, EMBO J. 2004; 23: 3206

LLO

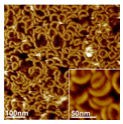


Environmental cues affect pore formation by LLO

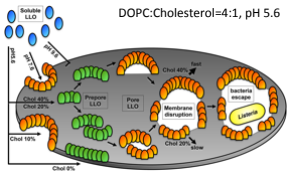


1,2-palmitoyl-sn-glycerophosphocholine (POPC):CHOL=1:1

Podobnik et al, Sci Rep. 2015,5:9623



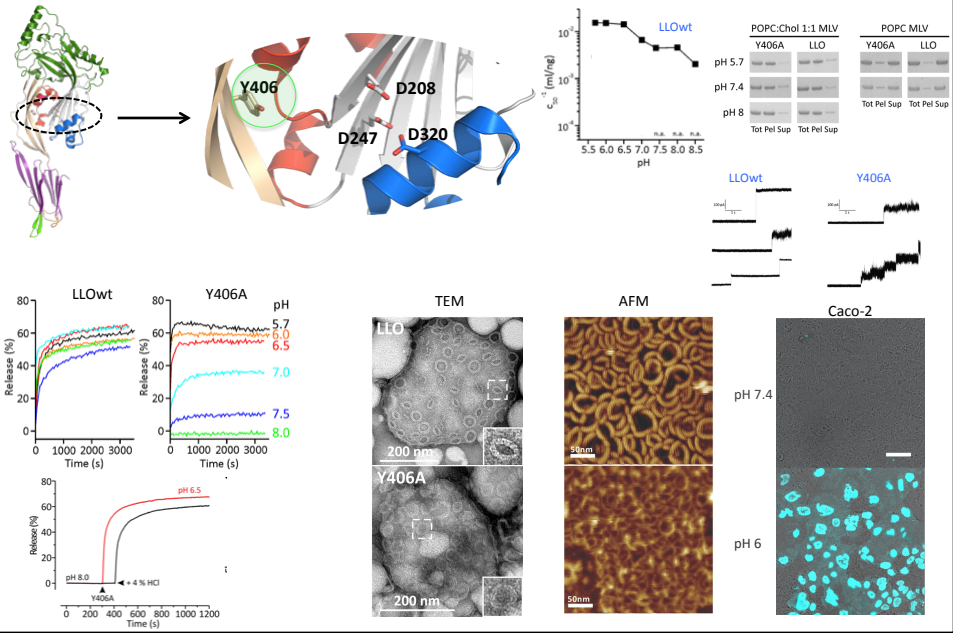
DOPC:Cholesterol=4:1, pH 5.6



Ruen et al, PLoS Pathog. 2016;12(4):e1005597

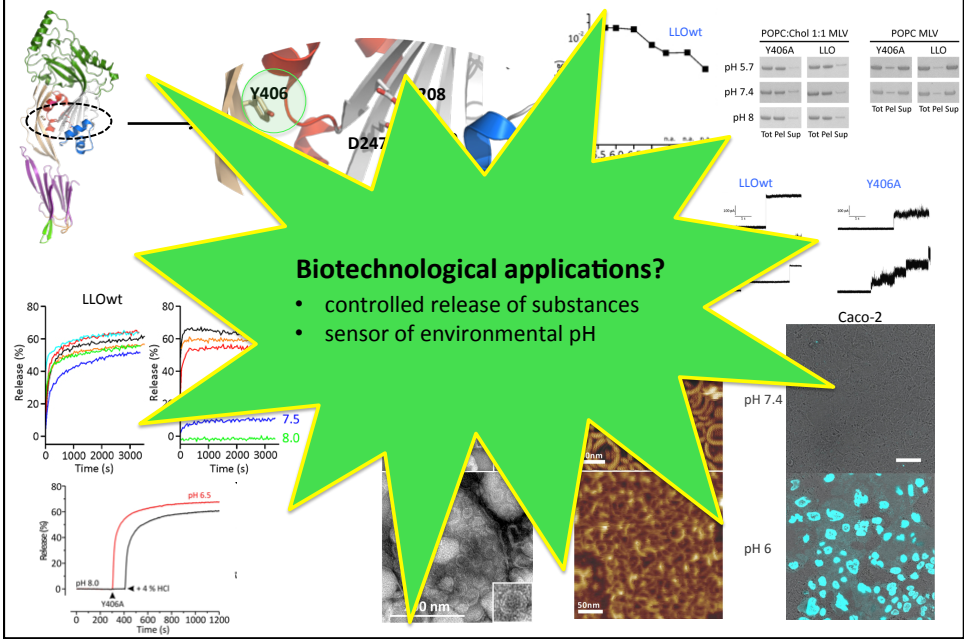
Engineering of pH responsive LLO

Kisovec et al, Sci Rep. 2017 Feb 8;7:42231

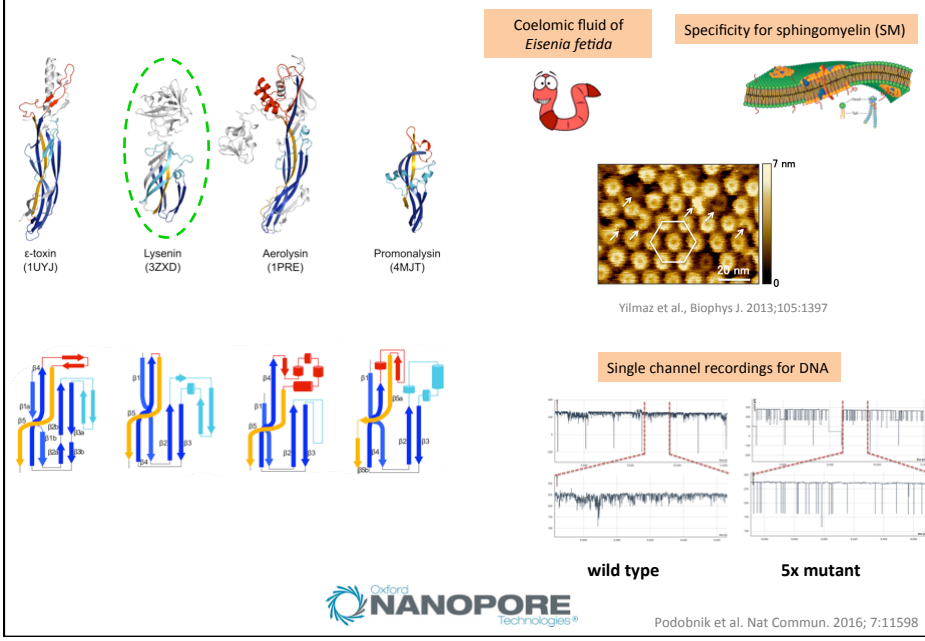


Engineering of pH responsive LLO

Kisovec et al, Sci Rep. 2017 Feb 8;7:42231



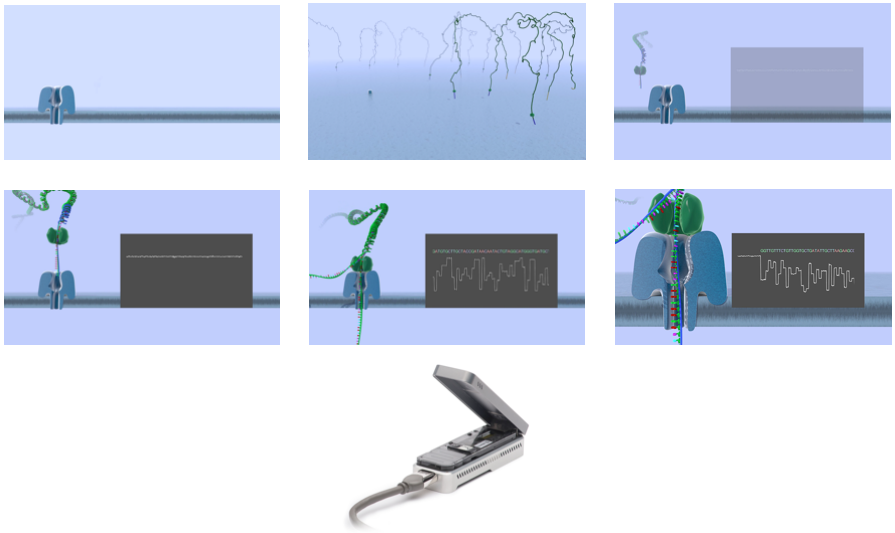
Lysenin: a member of Aerolysin family



Nanopore applications

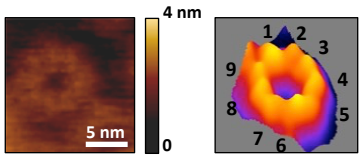
www.nanoporetech.com

RNA/DNA sequencing

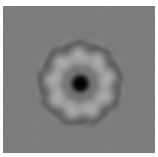


Lysenin pore is a nonamer

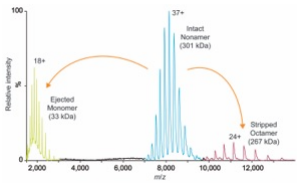
Podobnik et al., Nat Commun. 2016;7:11598



AFM

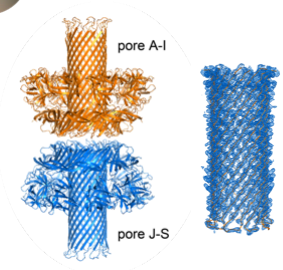


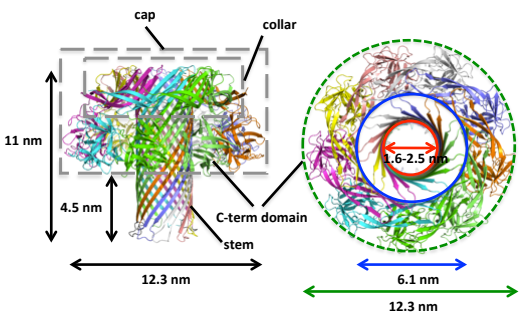
Electron crystallography
2D crystal - lysenin in liposomes containing SM



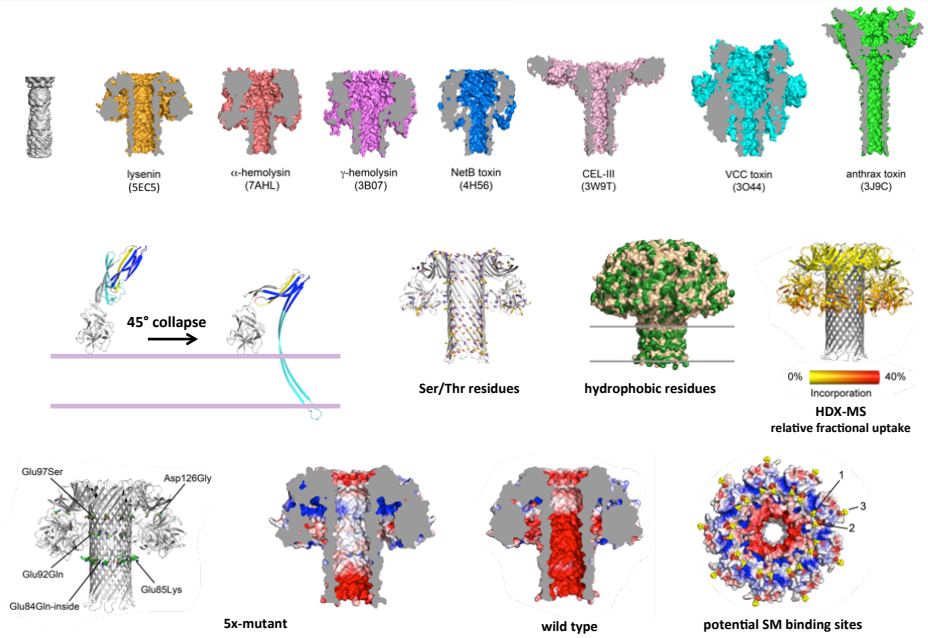
native MS

Crystal structure - 3.1 Å resolution

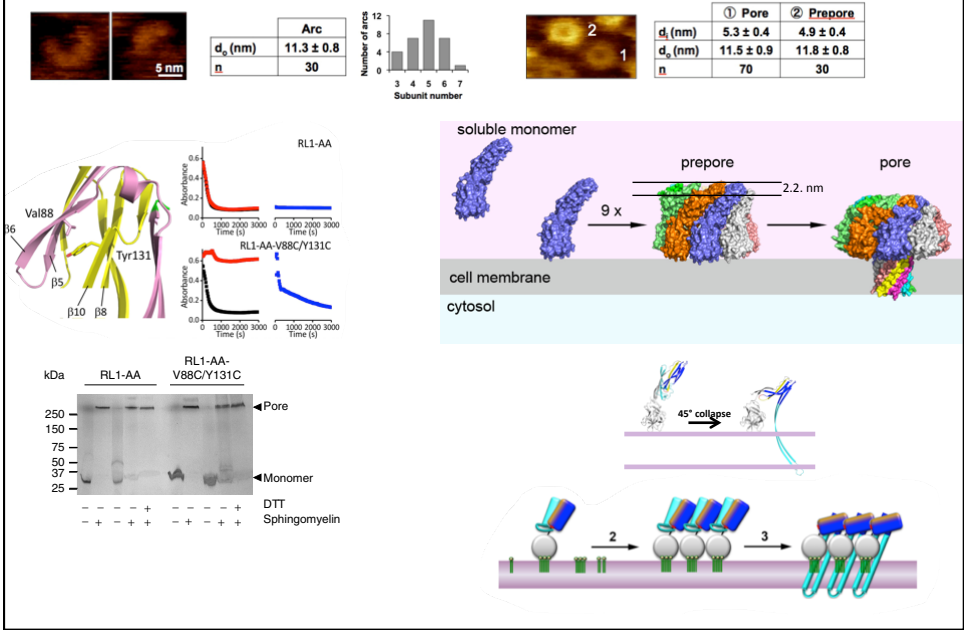




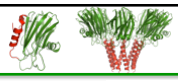
Lysenin pore properties



Mechanism of pore formation by lysenin: arcs, prepore, conformational change

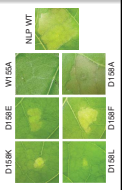


NLP proteins – eudicot plant toxins

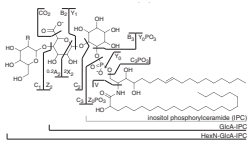


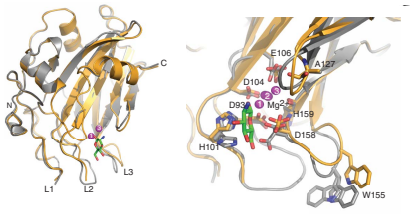
Lenarčič et al., Science 2017; 358, 1431–1434

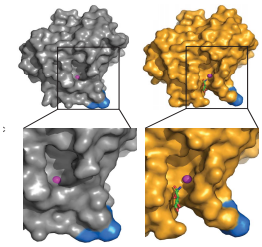
- Nep1-like proteins (Necrosis- and ethylene-inducing peptide 1) produced by bacterial, fungal and oomycete plant pathogens
- induces necrosis and ethylene production in leaves of **eudicot**, but not monocot plant species (*Great Irish Famine*, 1845-1849)
- NLPs target the outer leaflet of the plant plasma membrane



- determination of the receptor: glycosylinositol phosphorylceramide (GIPC) – plant sphingolipid
- determination of conformational changes in NLP proteins upon binding of sugar heads on GIPCs
- monocot GIPCs often carry three hexose units linked to IPC (series B GIPC), whereas eudicot GIPCs carry only two (series A GIPC)

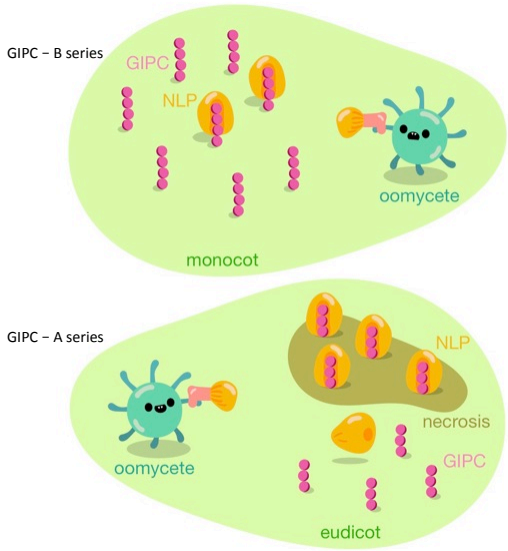


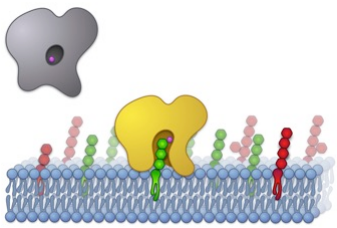




NLP proteins bind to monocots and eudicots but are toxic only to eudicots!

... and now we know why!





by Tea Lenarčič

Potato Virus Y (PVY): flexible filamentous virus

FAMILY: *Potyviridae*

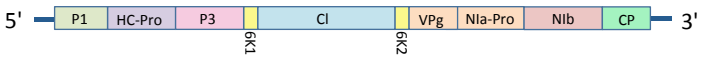
INFECTIONS: potato, tobacco, tomato, pepper

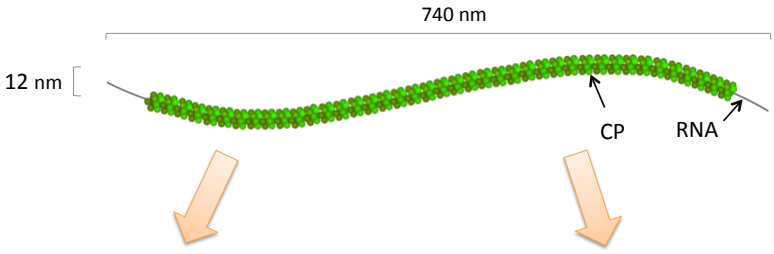
TRANSMISSION: aphids, mechanic

http://www.potatovirus.com/index.cfm/page/PVYinfo/Aphidinfo.htm

Scholthof et al., Mol. Plant. Pathol., 2011.

Potato Virus Y (PVY): flexible filamentous virus



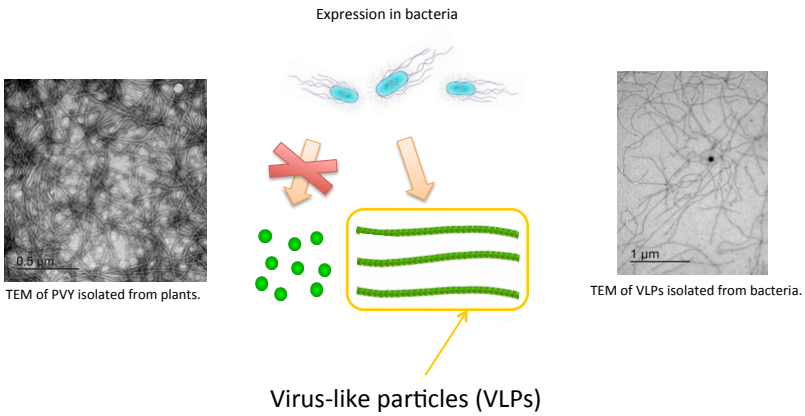


MECHANISM OF PATHOGENESIS

APPLIED SCIENCE

- pharmaceuticals
- vaccines
- biosensors
- template for nanomaterials
- nanoelectronics

Bacterial expression of CP protein leads to virus-like particle formation

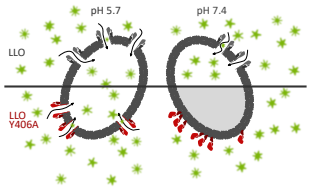


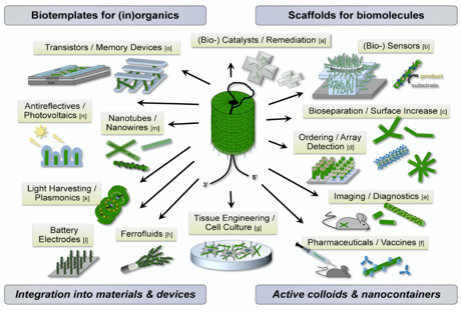
TEM of PVY isolated from plants.

TEM of VLPs isolated from bacteria.

Summary

- Nanosized objects – transmembrane pores and filamentous viruses
- Structures of monomers and complexes, mechanism of assembly, mechanism of action, interactions with other molecules →pathogenesis
- Nanobiotechnological applications





Koch C, et al: Bilstein J Nanotechnol. 2016; 7:613-29

Technical Operations

Improvement of therapeutic proteins by protein modification

Simona Jevševar
Lek Pharmaceuticals d.d., Ljubljana
DS BioProduction Mengeš
Manufacturing Science & Technology

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Agenda

- Biologics in modern medicine
- PEGylation
  - Why to PEGylate & What is changed?
- Half-life extension and *in vitro* BA dependence on PEG length and shape
- PEG-GCSF isoforms: *in vitro* BA, PK/PD behaviour
- General Manufacturing and Analytical strategy
- Comparison of process efficiency and analytical challenges for two N-terminally PEGylated proteins having low and high number of exposed Lys
- 1st and 2nd generation of filgrastim – differences in therapeutic behaviour

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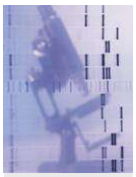
Biologics have revolutionized modern medicine – and will continue to do so

- Offer real hope for many unmet needs, particularly complex diseases
- Bind to specific targets within the body – simply not possible with other medicines
- Contribute significantly to improved survival rates, enhanced longevity, and better quality of life

DNA molecule decoded

1950s



Genetic code cracked

1960s

Basic biotechnology enabled

1970s

Genentech
AMGEN
biogen
Applied Biosystems

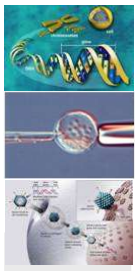
Commercial biotech firms founded

1980s

Enbrel
Remicade
Rituxan
PROCRIT EPREX
Aranesp
EPOGEN
Neulasta
Herceptin
AVONEX
AVASTIN (bevacizumab)

Leading biotech brands emerge

1990s to today



Human genome

Stem-cell research

Gene therapy

Today / future

Source: Company websites and annual reports Note: All trademarks, logos and pictures are the property of the respective owner

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Biologics are more complex than small molecules…

Aspirin®

Calcitonin

Monoclonal Antibody (IgG)

small chemical molecule	simple biologic	complex biologic
Molecular weight = 180 Daltons 0 amino acids	Molecular weight = 3,455 Daltons ~ 32 amino acids - w/o host cell modifications - produced in yeast, bacteria	Molecular weight = 150,000 Daltons ~ 1300 amino acids - w/host cell modifications (glycosolations, etc) - produced in mammalian cells

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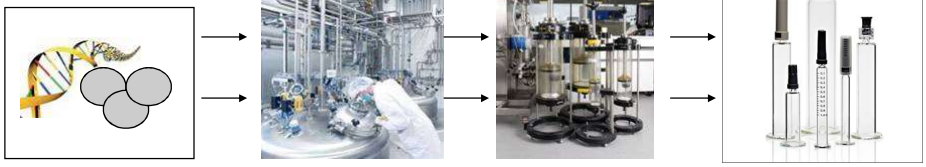
…and are produced from living organisms



Modify host cells (e.g., bacteria, mammalian yeast) to produce recombinant proteins

Grow cells under controlled conditions (fermentation)

Extract, refold, purify (downstream) – generate drug substance

Formulate to stable finished drug product (vial, syringe, cartridge)

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After development of a highly similar molecule, similarity is confirmed by clinical studies

1 Develop highly similar product

2 Confirm biosimilarity

Drug substance

Formulation / Drug product

GLP Tox.

Phase I

Phase III

Process validation

- **Technical development** aims to
  - **Achieve a "highly similar"**, to match originator molecule profile
  - **Match final dosage form** of originator
  - **Develop a high-yielding manufacturing** process
- **Clinical program**
  - Needs to be agreed with **health authorities,** e.g., indication, design, endpoints
  - **Scope** depends on **degree of similarity** to reference molecule
  - Should support **extrapolation** to non-studied indications, **interchangeability** & **commercial success**

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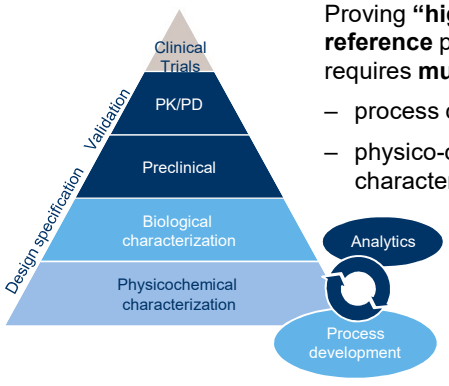
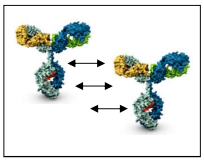
Target directed development is an iterative process, aiming for a highly similar structure



Proving **"highly similar" to reference** product often requires **multiple iterations** of

- process change
- physico-chemical characterization



"Highly Similar" Molecular Structure

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2nd Generation Biopharmaceuticals

Transforming Proteins into Protein Drugs with Improved Properties

- Majority of biopharmaceuticals – recombinant proteins with sub-optimal physico-chemical properties for **"Replacement"** and **"Antagonist" therapies**.
- Native proteins are very eficient and serve specific functions in the body and generally lack properties important for good medicines, i.e. solubility, stability, long elimination half-life, low immunogenicity, good expression in various host organisms,...

Amino acid engineering
From single mutations to large scale modifications
e.g., introduction/removal of glycosylation sites, alteration of protease sensitive regions…

Protein-protein fusion
(IgG, IgG1 Fc, albumin, transferrin, Hsp 65, antibodies or their fragments, fusion to homo-amino-acid polymer)

Advanced delivery systems
(liposomes, nanoparticles, microparticles)

Post-production modification: derivatization, polymer conjugation
(**PEGylation**, polysialylation, HESylation, fatty acid group derivatization)

Post-production modification: mAb for the next generations of biopharmaceuticals
(Antibody Drug Conjugates – ADCs)
mABs conjugation with toxins for targeted delivery ciljano dostavo toksinov v rakave celice

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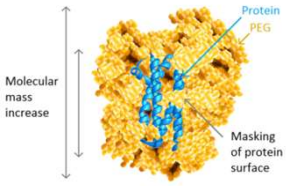
PEGylation - Why to PEGylate & What is changed?

PEGylation is the best established technology for improving protein therapeutic value with several products on the market

- ❑ Enhances bioavailability and prolongs plasma half life – *molecular size increase effect*
- ❑ Decreases immunogenicity – *shielding effect of PEG*
- ❑ Increases protease resistance – *shielding effect of PEG*
- ❑ Increases solubility & stability – *stabilization effect of PEG*
- ❑ Reduces aggregation and depot loss at injection sites – *stabilization effect of PEG*
- ❑ Benefits for the patient
  - More efficient therapy
  - Less frequent administration



.. when PEG is covalently attached to the protein, physicochemical and biological properties of protein are changed!

- ❑ **Biological properties changes:** biological activity *in vitro*/*in vivo*, absorption rate and bioavailability, biodistribution, PK/PD
  - consequences for efficacy of the final drug...
- ❑ **Physicochemical properties changes**: Mw, solubility, hydrodynamic radius, viscosity
  - consequences for Analytics, DSP, formulation, injectability

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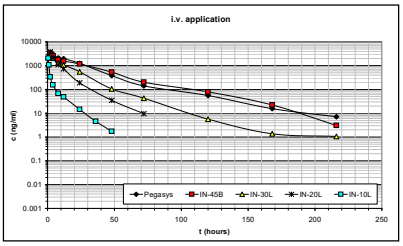
Half-life extension and *in vitro* BA dependence on PEG length and shape

Substance IFNα-2b	$t_{1/2}$ (hours) in rats		Biol. activity *in vitro* (%) *(ratio between conjugates)*
	s.c. application	i.v. application	
IFNα-2b	0.8±0,1	-	100
IFN-PEG-10L	7.1±0,1	7.3	13.9 *(12.6x)*
IFN-PEG-20L	18.2±1,8	10.5	8.2 *(7.4x)*
IFN-PEG-30L	25.0±0,7	19.9	6.3 *(5.7x)*
IFN-PEG-45B	52.6±2,3	22.0	1.7 *(1.5x)*
PEGASYS (PEG-40B)	59.6±7,5	23.9	1.1 *(1x)*



The *in vitro* activity determined by cell-based bioassays for PEGylated proteins is not predictive for the *in vivo* therapeutic effect, because of the phenomenon that the major effect of PEGylation is steric hindrance caused by flexible PEG chain and not conformational changes. (*Bailon, P., Won, C. Y., PEG-modified biopharmaceuticals. Expert. Opin. Drug Deliv. 2009, 6, 1-16)

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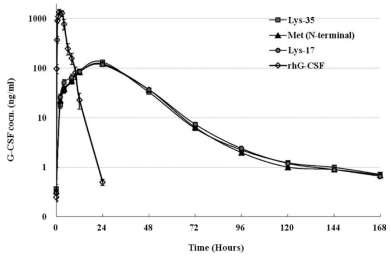
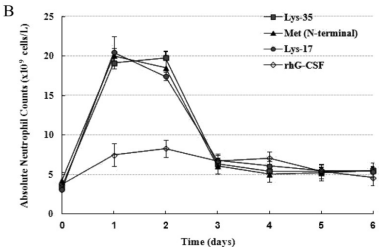
PEG-GCSF isoforms

In vitro BA, PK/PD behaviour (adopted from Kang JS& Lee KC, *Biol. Pharm. Bull.* 36(7) 1146–1151 (2013))





Substance hG-CSF	$t_{1/2}$ (hours) in rats	Biol. activity *in vitro* (%)
	s.c. application	
hG-CSF	2.9±0.1	100
hG-CSF-PEG-23 (Lys35)	19.1±0.3	~20.1
hG-CSF-PEG-23 (Lys17)	21.2±0.7	~15.3
hG-CSF-PEG-23 (N-terminal)	18.9±0.2	~37.4

Significant differences between individual positional isomers of mono-PEGylated rhG-CSF in *in vitro* biological activities (PEGylation site dependent). However, all three isomers exhibit comparable *in vivo* pharmacokinetic and pharmacodynamic properties.

Similar own experience with attachment of 20 kDa PEGL to hG-CSF by N-terminal and random PEGylation. Similar PK/PD behaviour of both (smaller difference in *in vitro* BA).

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PEGylated proteins: Manufacturing and Analytical strategy

Generally, highly purified proteins are subjected to PEGylation due to:

- PEGylation step increase Mw contributing to large masking effect of PEG influencing analytical resolution
- HCP can be PEGylated, thus should be removed prior to PEGylation
- Cost of PEG reagents also contributes to the approach to test protein thoroughly prior to PEGylation

Several PEGylated product produced from already approved 1. generation drugs; PEGylation used to improve their characteristics and for life cycle management

- *(PEGIntron® (IFN-α2b); PEGASYS® (IFN-α2a); Neulasta® (G-CSF), MIRCERA® (epoetin-β))*
- Highly purified proteins and well characterized proteins starting point for PEGylation in these cases
- Several release tests at protein level (e.g., HCP, residual DNA) and no need to repeat them after PEGylation

Extended testing at protein (intermediate) level is generally performed

- RP-HPLC and SE-HPLC purity
- *In vitro* bioactivity
- Residual solvents
- HCP (release test for PEGylated DS at protein level prior to PEGylation)
- Residual DNA (release test for PEGylated DS at protein level prior to PEGylation)

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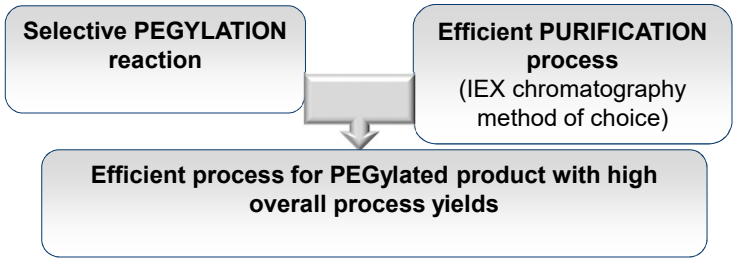
How to develop PEGylated products?



Well defined single PEG-conjugates are preferentially desired.

- Two classical site-specific PEGylation reactions with high reaction specificity:
  - N-terminal PEGylation using PEG-CHO reagent
  - Thiol PEGylation using PEG-Maleimide
- Amber technology for site-specific PEGylation reactions via unnatural amino acids

By variation of PEGylation parameters (protein concentration, molar excess of PEG reagent, reaction time, temperature) PEGylation specificity and yields can be increased.

For planning of experiments and evaluation of results general factorial design is used (Design-Expert Software 7.0).

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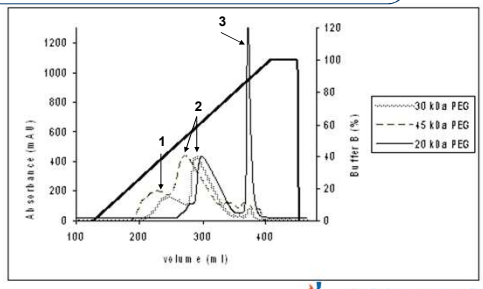
How to develop PEGylated products?

Selective PEGYLATION reaction

Efficient PURIFICATION process (IEX chromatography method of choice)

Efficient process for PEGylated product with high overall process yields

Comparison of preparative CEX separations of IFN pegylation mixtures prepared with various mPEG-aldehyde reagents of different lengths and shapes on TSK-gel SP-5PW column (Tosoh Bioscience, Japan). Peak 1 designates higher-PEGylated IFN forms, 2 mono-PEGylated IFN forms and 3 un-PEGylated IFN.



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Analytical techniques

Characterization of PEGylated proteins

Chromatographic methods Detection mode: UV/VIS Fluorescence Corona CAD	- Reverse Phase chromatography - Ion exchange chromatography - Size exclusion - SEC UV/RI MALLS - Peptide mapping	- Purity and Content - different degree of PEGylation, unpegylated protein and tipically oxidized and deamidated variants - Purity, Identity and Charge heterogeneity in PEG-protein conjugate - Size/degree of PEGylation - Mass distribution of PEG.protein conjugates (as well as mass distribution of PEG and protein) - Identification of PEGylation sites; (detection of oxidized and deamidated species when RPC is not able to detect them)
Gel Electrophoresis Detection mode: -Coomassie -Iodine staining -Silver staining	- SDS-PAGE - Native gel electrophoresis	Conjugate size, Purity - dimers, multyPEG species, unpegylated protein, degradation products,…
Spectroscopic methods	- UV/VIS - Fluorescence - Circular Dichroism - NMR - MS - (MALDI-TOF) - Surface Plasmon Resonance-BIAcore	
Physico-chemical characterization tools	- Dynamic Light Scattering Detector - UV/VIS spectrophotometer with Peltier - Isothermal Titration Calorimetry (ITC) - DSC	- Hydrodynamic radius - Melting point - Conjugate – target interaction - Termodynamic stability
ELISA	- antiProtein ELISAs - antiPEG ELISAs	Measurement of PEG/Prot conjugates in serum (determination of PK profiles)
Bioassays	- *In vitro* cell line assays	Biological activity of PEGylated proteins

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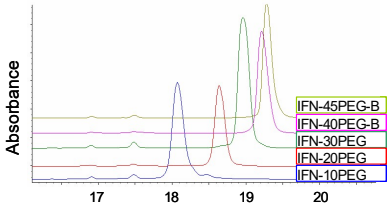
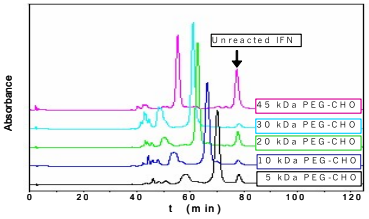
Analytical techniques
Characterization of PEGylated proteins

Series of N-terminally PEGylated IFNalpha was prepared and characterized.

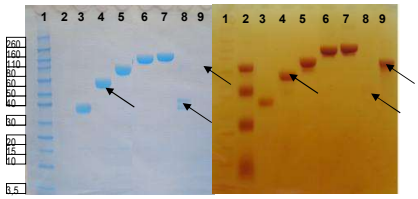
RPC analysis of various PEG- IFN conjugates.



CEX analysis of PEGylation mixtures of IFN with various PEG reagents of different lengths (5, 10, 20, 30 and 45 kDa).



SDS-PAGE of PEG standards, PEG-IFN conjugates and ovalbumin – simply blue stained (a), iodine stained (b).



Lane 1: Novex Sharp Mw standard
Lane 2: PEG MIX standard (5, 10, 20, and 30 kDa)
Lane 3: IN-10L/S3
Lane 4: IN-20L/S2 (~40 kDa)
Lane 5: IN-30L/S2
Lane 6: IN-40B/S1
Lane 7: IN-45B/S2
Lane 8: Ovalbumin (~40 kDa)
Lane 9: 40 kDa PEG (~40 kDa)

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Analytical techniques
Characterization of PEG reagents

Chromatographic methods Detection mode: UV/VIS Fluorescence Corona CAD	- RP-HPLC	- Purity after derivatization with p-ABA (UV/VIS and fluorescence) - Purity – Corona CAD (detection possible without derivatization, chaged aerosol detection mode) – no difference between activated and nonactivated impurities
	- SE-HPLC	- Size, Purity of PEG – derivatization needed for UV/VIS and fluorescence, while Corona CAD enables detection without derivatization
	- SEC UV/RI MALLS	- Mass distribution of PEG reagent
Gel Electrophoresis Detection mode: -Iodine staining	SDS-PAGE	- PEG Size, Purity
Spectroscopic methods	- NMR	- quality of PEG (identity, terminal activity)
	- MS - (MALDI-TOF)	- Mw of PEG

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Analytical techniques
Characterization of PEG reagents

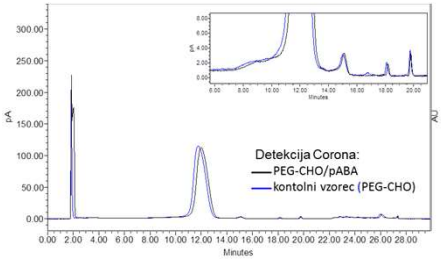

Figure A: Derivatization of PEG-CHO with PABA – CAD detection

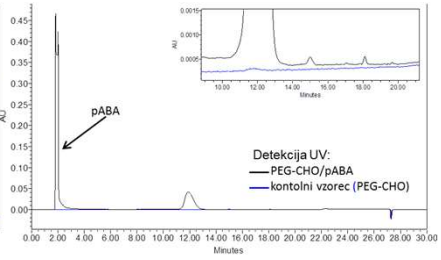

Figure B: Derivatization of PEG-CHO with PABA – UV detection

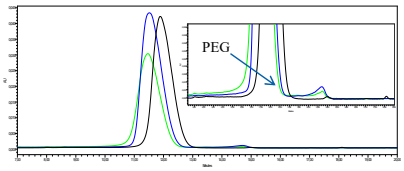

Figure C: Chromatograms of 30 kDa PEG reagents using UV detection; PEG 1 (black trace), PEG 2 (blue trace, PEG 3(green trace)

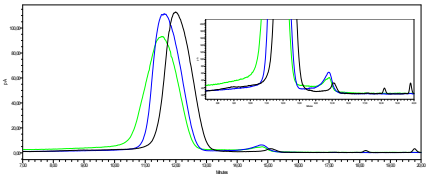

Figure D: Chromatograms of 30 kDa PEG reagents using CADdetection; PEG 1 (black trace), PEG 2 (blue trace), PEG 3 (green trace)

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Comparison of process efficiency for two N-terminally PEGylated proteins

- LA-EP2006: N-terminal attachment of 20 kDa PEG-aldehyde
- BVS857: N-terminal attachment of 30 kDa PEG-aldehyde

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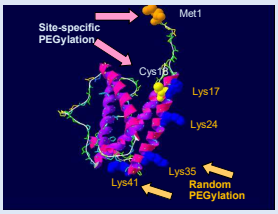
LA-EP2006 molecule: mono-PEGylated EP2006

molecule	Mw (Da)
EP2006	18800
LA-EP2006	40000

$CH_3O(CH_2CH_2O)n\text{-}CH_2CH_2CH_2\text{-}$ NH of Met

n is approx. 477 for 20 kDa PEG

PEGylation site (99%): **N-terminus** (H2N-Met)



Only two Lys exposed for potential PEG attachment, reaction specificity for N-terminus is very high leading to more than 75% of monoPEGylated molecules in PEGylation mixture.

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BVS857 molecule: mono-PEGylated BVS857 peptide

molecule	Mw (Da)
BVS857pep	11219
BVS857	41219

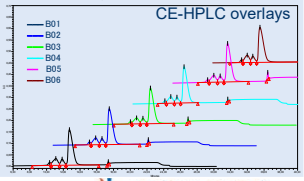
$CH_3O(CH_2CH_2O)n\text{-}CH_2CH_2CH_2\text{-}$ NH of Gly or Lys

n is approx. 690 for 30 kDa PEG

Primary PEGylation site (min 65%; real range 71-75%): **N-terminus** (H2N-Gly)

Secondary PEGylation sites (max 35%; real range 25-29%): **Lys 26, 64, 67, 80, 83, 88, 98**

Many exposed Lys decrease specificity of reductive alkylation directed to N-terminus. Fast proceeding of reaction to higher-PEGylated species leads to lower amount of desired mono-PEGylated form – only cca. 45% of monoPEGylated molecules in PEGylation mixture.



Final BVS857 mixture of different positional isoforms. Consistency of PEG pattern is controlled by CE-HPLC.

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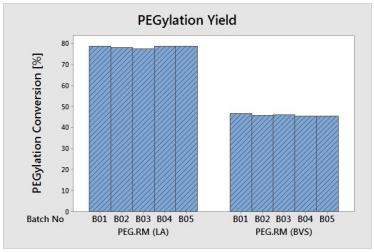
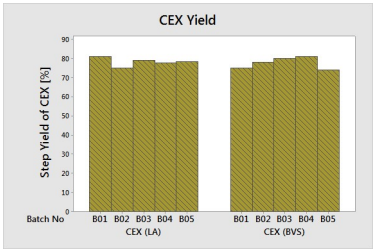
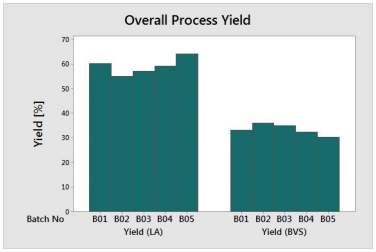
Large scale LA-EP2006 & BVS857 DS batches

Overall process and step yields







- Process steps yields and overall process yield for PEGylation part are consistent for both proteins – difference in PEGylation conversion refelcted in Overall Process Yield.

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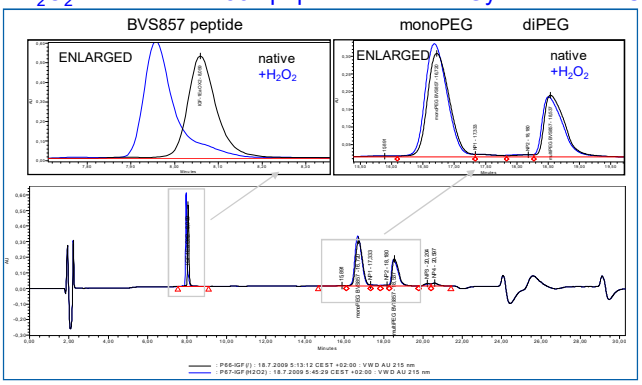
BVS857 DS

Analytical resolution before and after PEGylation

BVS857 peptide → PEGylation → RPC of PEGylation mixture

H_2O_2-oxidized BVS857 peptide → PEGylation → RPC of PEGylation mixture



RPC analysis

- BVS857 peptide: significant shift in ret. time.
- PEG species: non-significant shift in ret. time.
- PEG:protein = 2.7:1
- Oxidation in PEGylated sample not detected by RPC, pepmap is needed for detection of oxidized species in BVS857.

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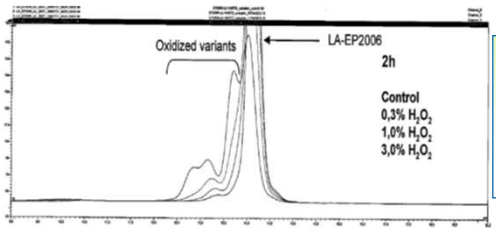
LA-EP2006 DS and IFN-ß-1b
Analytical resolution before and after PEGylation

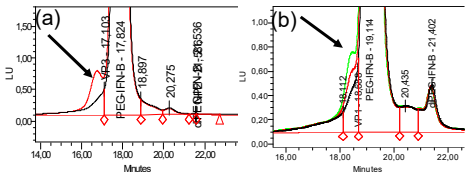
Figure E: LA-EP2006; overlay of control and oxidised samples.



RPC analysis

- PEG:protein = 1:1
- Oxidized LA-EP2006 variants are detected. RPC is suitable for detection of oxidized species in LA-EP2006.

Figure F: Oxidized 20L-IFN-ß-1b (a) and 30L-IFN-ß-1b (b) are detected as a prepeak shoulder(s)



RPC analysis

- PEG:protein (IFNbeta) = 1:1 (20 kDa); 1.5:1 (30 kDa)
- Oxidized PEG-IFNbeta variants are detected by RPC for PEG20L&30L. Resolution is lost for PEG40L&40B.
- **The separation is better when smaller PEG is attached.**

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filgrastim versus pegfilgrastim
1st generation vs 2nd generation

filgrastim and pegfilgrastim

What do they bring for the patients?

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filgrastim versus pegfilgrastim
1st generation vs 2nd generation

Filgrastim: 1st generation of hG-CSF
recombinant hG-CSF produced in *E.coli*

Pegfilgrastim: 2nd generation of hG-CSF
N-terminally PEGylated hG-CSF - half-life extension through PEGylation

Substance hG-CSF	$t_{1/2}$ (hours) s.c. application	Biol. activity *in vitro* (%)
hG-CSF	2-4	100
Neulasta (hG-CSF-PEG-20L)	~44	~45

PEG attachment and hydrodynamic radius increase:
- 10 kDa PEGL to 20 kDa protein - hydrodynamic radius ↑ above protein of Mw 160 kDa
- 45 kDa PEGB to 20 kDa protein - hydrodynamic radius ↑ above protein of Mw 440 kDa

Renal clearance is almost blocked, removal through neutrophil mediated clearance.

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filgrastim versus pegfilgrastim
1st generation vs 2nd generation*

Medicine	filgrastim	pegfilgrastim
Protein	rh Met-G-CSF	rh Met-G-CSF
PEGylation site	/	N-terminus (20 kDa linear PEG aldehyde)
Mw	approx. 19 kDa	approx. 40 kDa
Dose	Vials or prefiled syringes, 300 µg or 480 µg protein / dose	prefiled syringes, 6 mg protein /dose
Final farmaceutical formulations	Solution w/o preservatie, pH 4.0 acetate, sorbitol, Na, Tween 80, WFI	Solution w/o preservatie, pH 4.0 acetate, sorbitol, Na, Tween 80, WFI
Administration regime	Daily (up to 2 weeks after each chemotherapy cycle until ANC reaches 10,000/mm^3)	One dose per chemotheryphy cycle
Elimination half-life $T_{1/2}$	approx. 3.4 h	approx: 44 h (range: 15-80h)
Clearance mehansm	Renal filtration + neutrophil mediated clearance	neutrophil mediated clearance
Efficacy (average of severe neutropenia in days)	Study 1 (n=157): 1.6 Study 2 (n=310): 1.6	Study 1 (n=157): 1.8 Study 2 (n=310): 1.7

*data in table refer to the originator products

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Conclusions

- Traditional PEG reagents generated several successful PEGylated therapeutics with reduced administration frequency, which have been safely used for many years.
- Longer PEG chain prolongs elimination half-life more; balance between half-life prolongation and other characteristics of PEG should be considered as well (accumulation, viscosity, analytical resolution...)
- PEGylation process in large scale is fully manageable and its performance is comparable to other process steps in purification of proteins.
- Overall process yields are largely depended on selectivity of PEGylation reaction and lost of conversion always means reduction of process yields.
- Due to masking effect of PEG influencing analytical resolution combined approach with testing of final PEG-conjugate and extensive testing of protein intermediate is needed in large scale production to ensure consistent quality and complete information.

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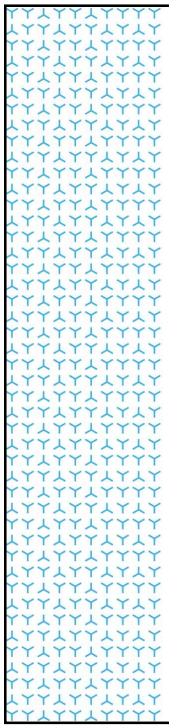

Thank You

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Back up slides

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Marketed PEGylated Biopharmaceuticals

Name	Company	Original protein	Therapeutic indication	Engineering rationale	Year to market
Adagen	Enzon	Bovine Adenosine Deamidase	Severe combined immunodefficiency (SCID)	Increased serum half-life	1990
Oncaspar® (Pegaspargase)	Enzon	Asparaginase	Acute lymphoblastic leukemia	Increased serum half-life, less alergic reactions	1994
PEG-Intron® (PEGIFN-α2b)	Schering-Plough / Enzon	IFN-α2b	Hepatitis C	Increased serum half-life	2001
Pegasys® (PEGIFN-α2a)	Hoffmann-La Roche	IFN-α2a	Hepatitis C	Increased serum half-life	2002
Neulasta® (pegfilgrastim)	Amgen / Nektar	G-CSF	Neutropenia	Increased serum half-life	2002
Somavert® (Pegvisomant)	Pfizer / Nektar	hGH mutein	Acromegaly	hGH–receptor antagonist	2003
Certolizumab pegol (Cimzia)	UCB	anti TNF Fab	Reumatoid arthritis and Crohn's disease	Increased serum half-life	2008
MIRCERA® PEGylated epoetin-β	Hoffmann-La Roche,	epoetin-β	anemia associated with chronic renal failure	Increased serum half-life	2007
Krystexxa® (pegloticase)	Savient Pharmaceuticals	recombinant mammalian urate oxidase	Chronic gout	Reduced immunogenicity and Increased serum half-life)	2010
Plegridy® (peginterferon beta-1a)	Biogen Idec Ltd	interferon beta-1a	Relapsing-remitting form of Multiple sclerosis (MS)	Increased serum half-life	2014
Macugen® or Macuverse® (pegaptanib)	Pfizer	anti-VEGF aptamer (an RNA oligonucleotide)	treatment of ocular vascular disease		2004

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