Polymeric polypeptides

How structure influence functional properties
of branched chain polymeric polypeptides ?

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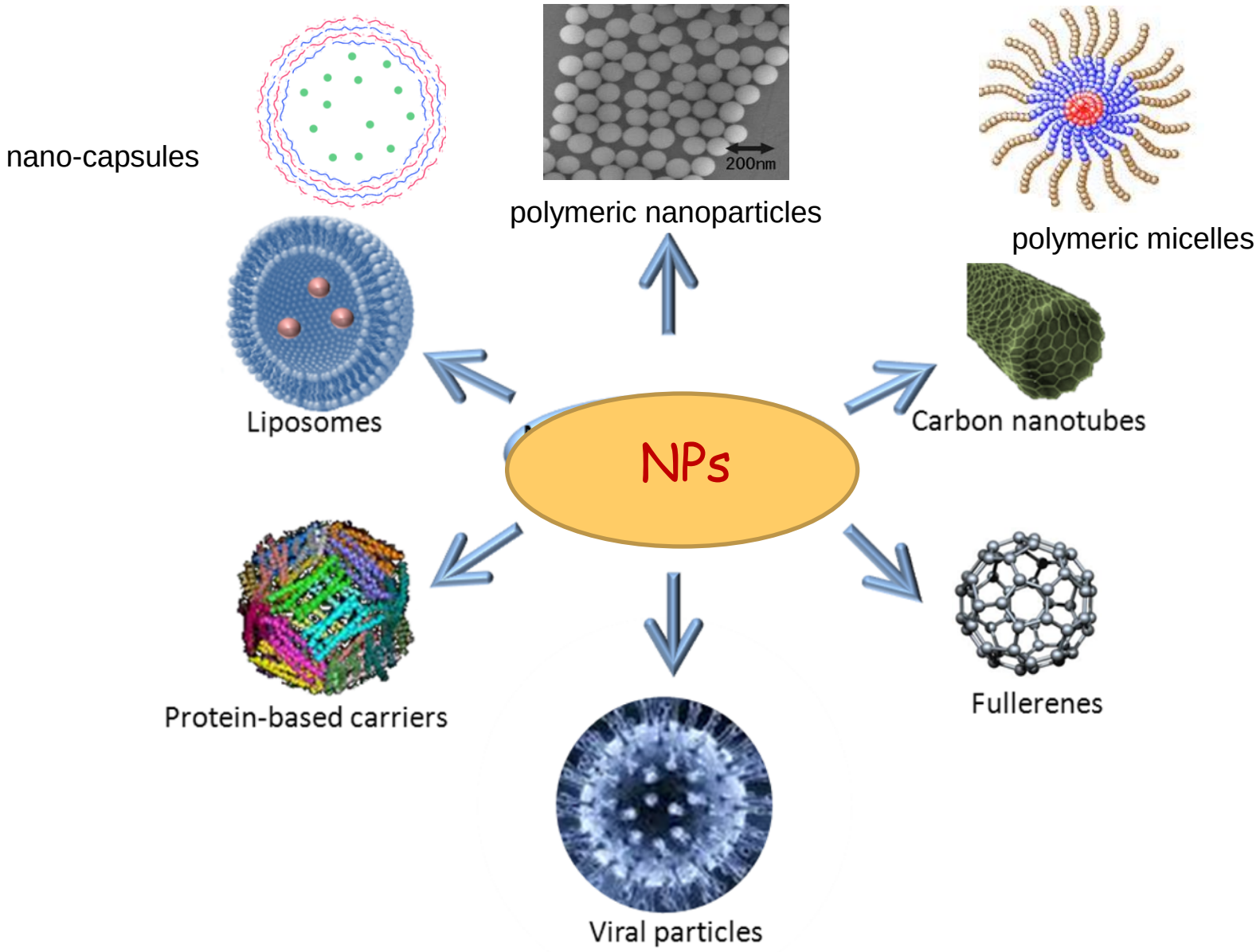
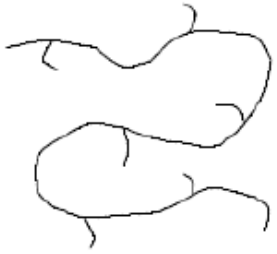
Nanoparticle based carriers for biomedical applications

Ryvolova, M. et al. *Sensors* 12: 14792-14820 (2012)

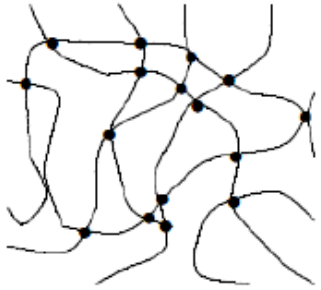


Branched polymers

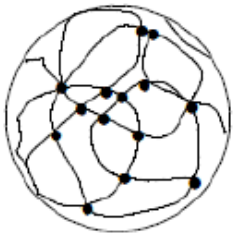


short chain branching

highly branched

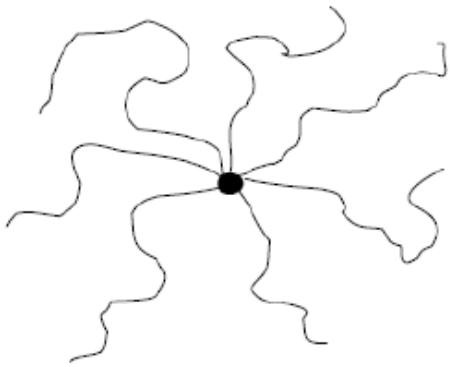


networks

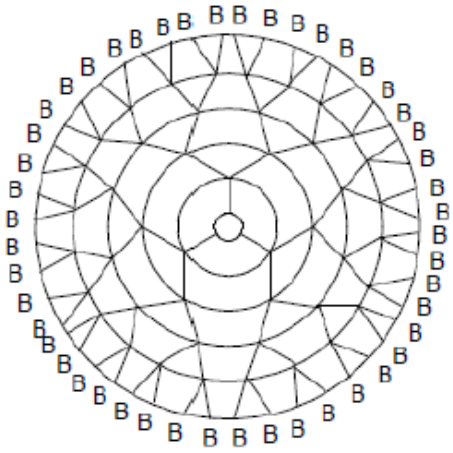


microgels

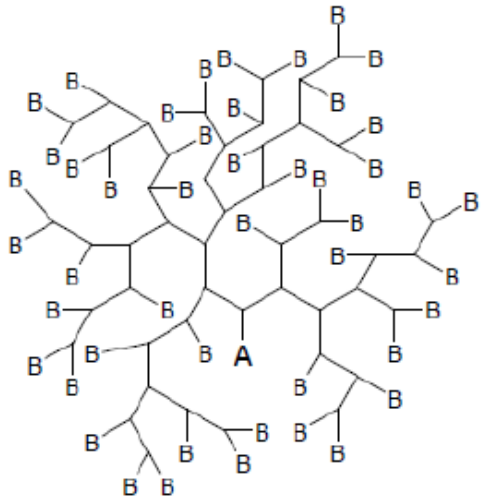
comb



stars



dendrimers



hyperbranched polymers

Polymer Chemistry - Dendrimers and Hyperbranched Polymers, chemie.unibas.ch

Conjugates with polymeric polypeptides

Synthetic antigens

- peptide vaccine
- immunodiagnostics

HSV gD glycoprotein

tuberculin proteins

mucin glycoprotein(s)

Drug/reporter targeting

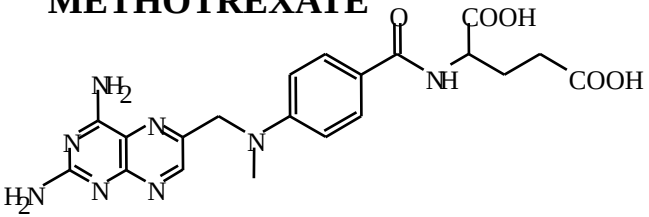
- increased specificity
- prolonged effect

antitumor drugs
- daunomycin
- vinblastin

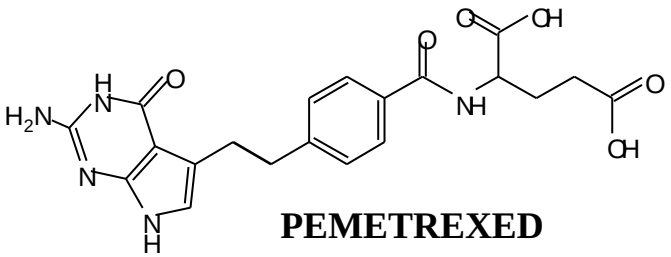
antimicrobials
- Leishmania don. (e.g. methotrexate)
- *M. tuberculosis* (e.g. isoniazid)

Drug, epitope, reporter molecule

METHOTREXATE

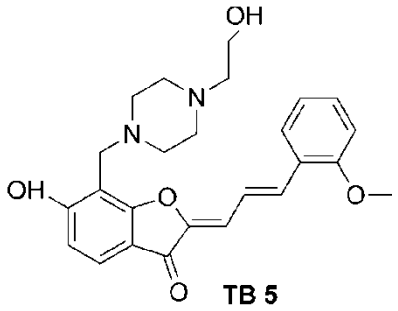


Hudecz F. et al. *Bioconjugate Chem.* **4:** 25 (1993)
Kóczán Gy. et al. *Bioconjugate Chem.* **13:** (2002)



PEMETREXED

Miklán Zs. et al.
J. Peptide Sci. 17: 805 (2011)



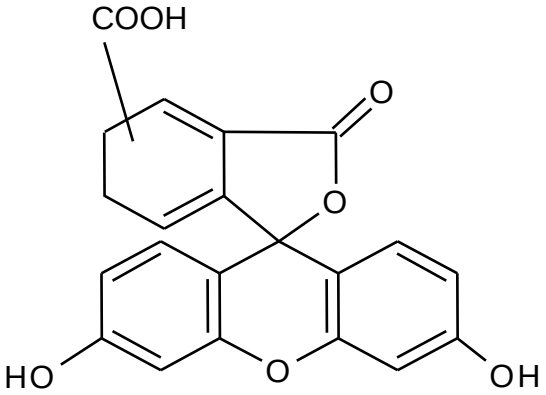
TB 5

Horvati, K.. et al.
Bioconjugate Chem. 22:981 (2012)

D-Trp-*D*-Cpa-*D*-Trp-Ser-Tyr-*D*-Lys-Leu-Arg-Pro-*D*-Ala

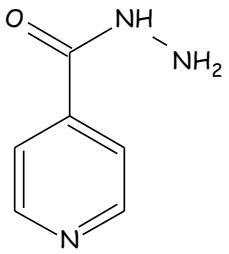
GN-RH ANTAGONIST, MI-1544

Mező, G. et al.*Bioconjugate Chem.* **7:** 642 (1996)
Vincze, B. et al. *J. Cancer Res. Clin. Onc.* **120:** 578 (1994)



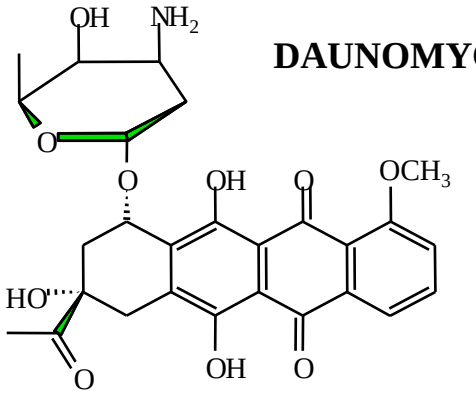
5(6)-CARBOXYFLUORESCEIN

Szabó R. et al. *Bioconjugate Chem.* 19: 1078 (2008)
Bánóczi Z. et al. *Bioconjugate Chem.* 19: 1375 (2008)



ISONIAZID

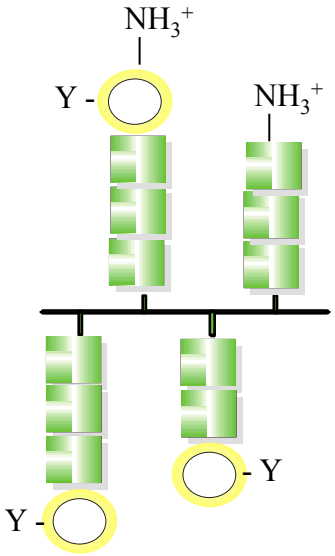
Horvati, K.. et al.
J. Peptide Sci. 15:385 (2009)



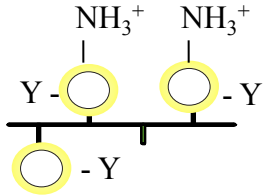
DAUNOMYCIN

Bánóczi Z. et al. *Archivoc* 143 (2008)
Miklán Zs. et al. Biopolymers 92:489 (2009)
Szabo R. et al. *BBA* 1798: 2209 (2010)

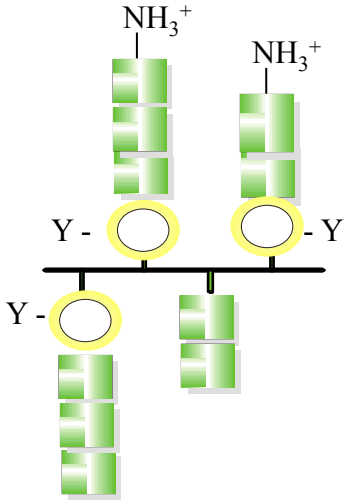
Branched polypeptides



XAK, poly[Lys(X_i-DL-Ala_m)]



X_iK, poly[Lys(X_i)]



AXK, poly[Lys(DL-Ala_m-X_i)]

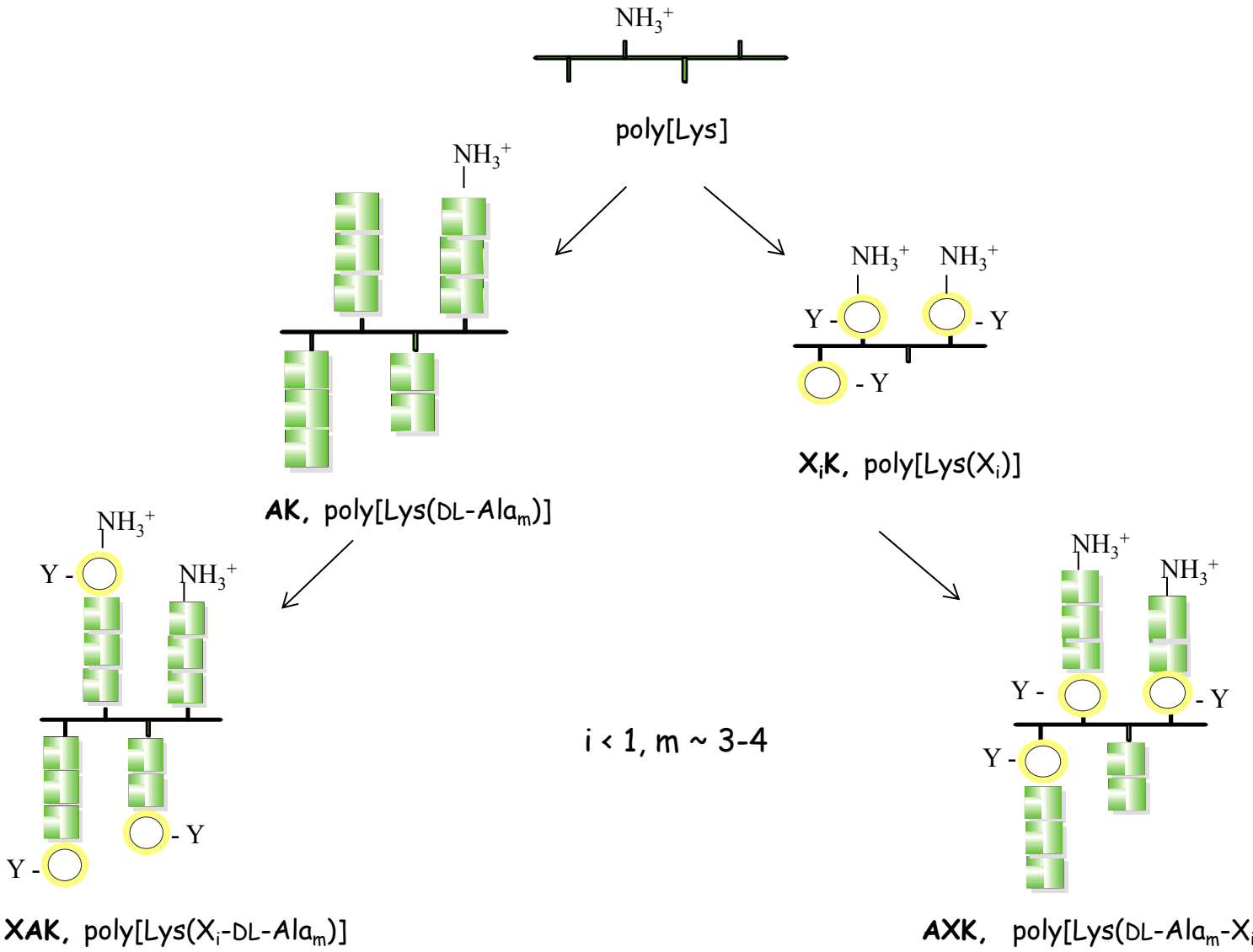
Hudecz,F.: *(Eds.: Agelli, A., Boden, N., Zhang, S.) Kluwer Academic Publisher (2001), pp. 139-160*

Hudecz, F., Kóczán, Gy., Reményi, J.: *(Eds.: Kéri, Gy., Tóth, I.) Taylor and Francis Group (2003) pp. 553-578*

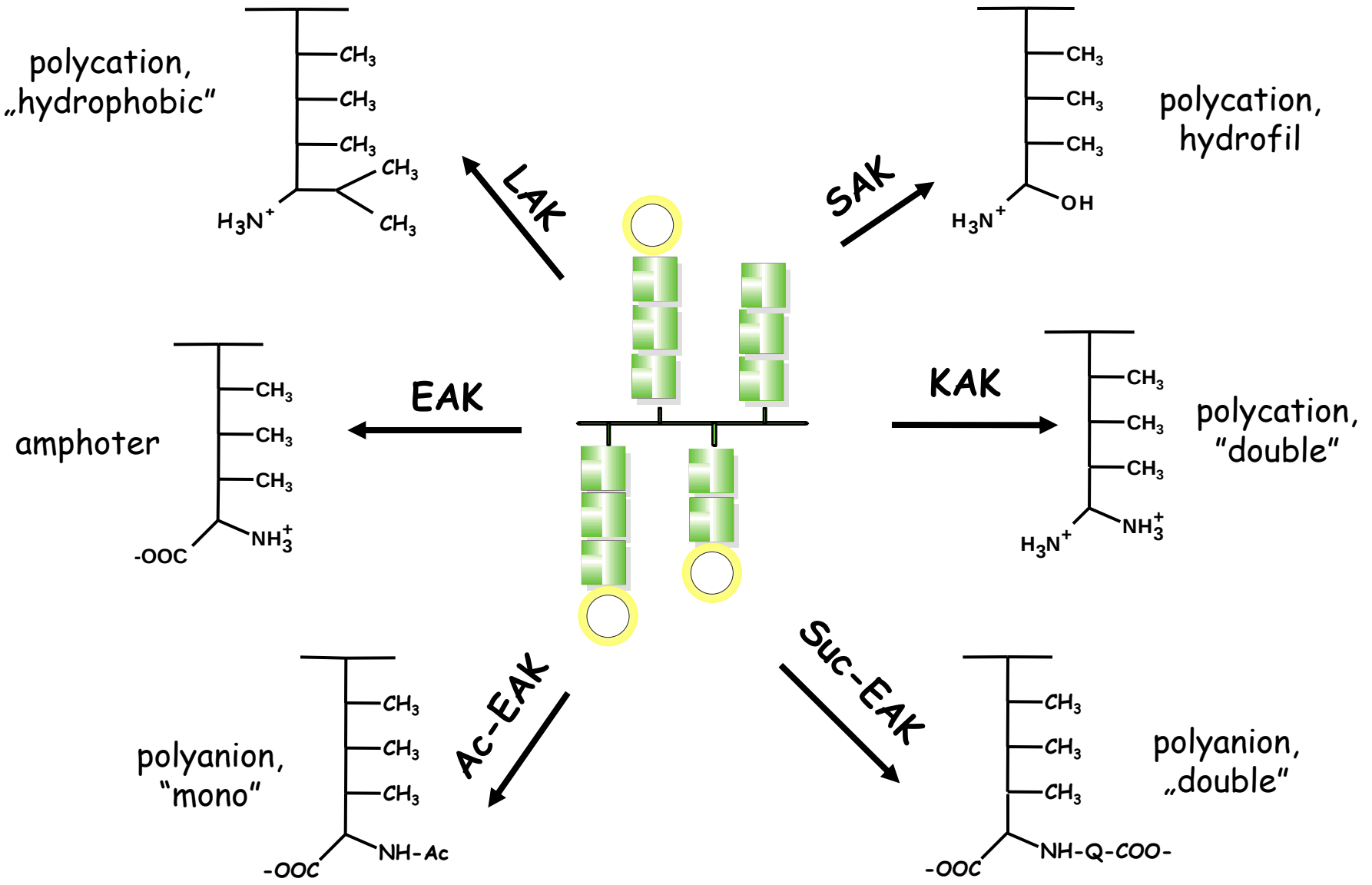
Sebestyén, M., Szabó, R., Kőhidai, L., Pállinger, É., Mező, G., Kóczán, Gy., Hudecz, F.: Structural Chemistry, 28: 527-536 (2017)

Szabó, R., Sebestyén, M., Kóczán, Gy., Orosz, Á, Mező, G., Hudecz, F.: ACS Combinatorial Science 19: 246-254(2017)

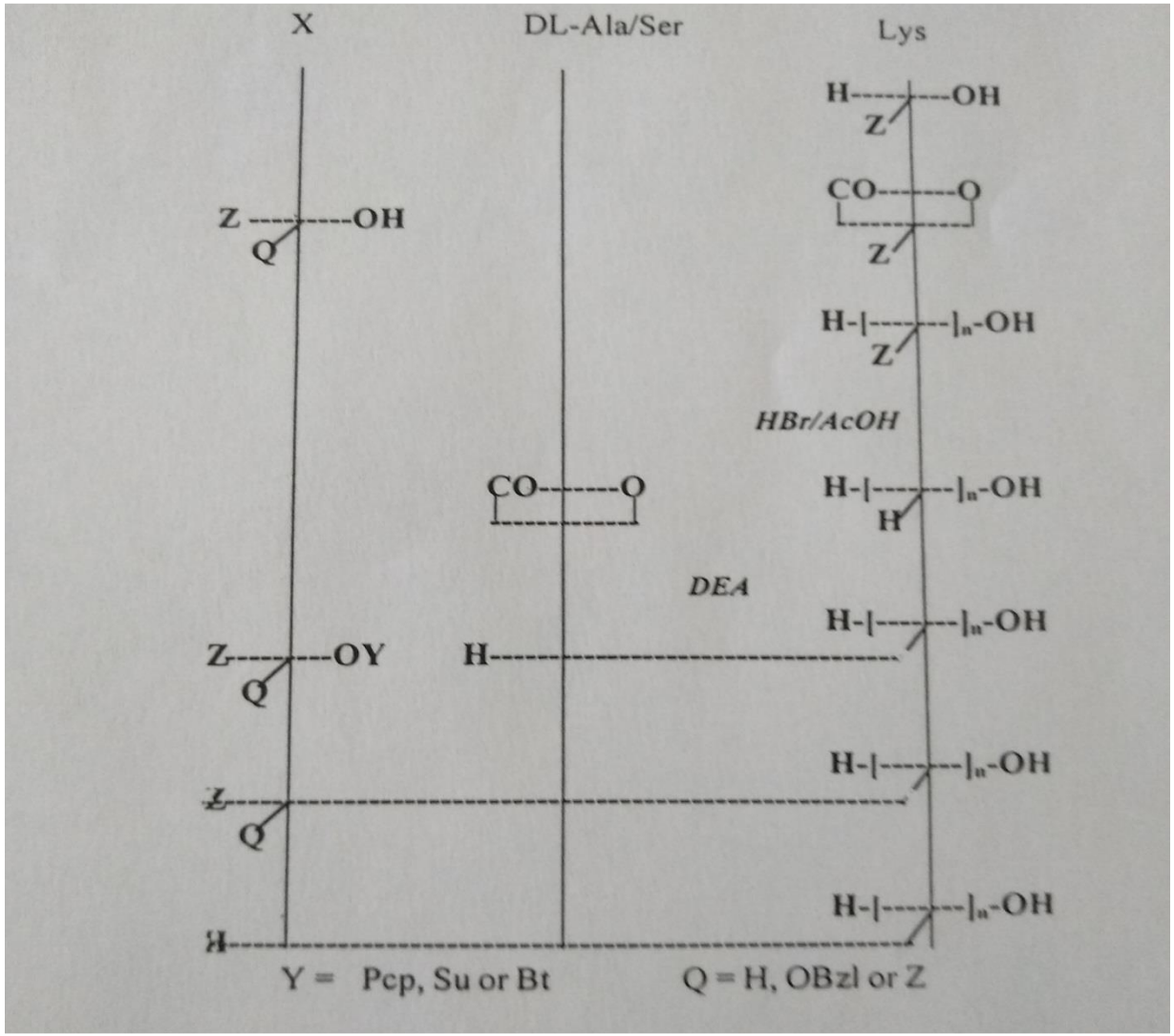
XAK, X_iK and AXK type branched polypeptides: synthesis

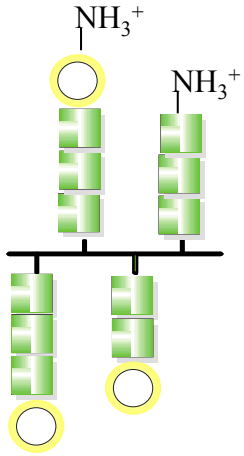


XAK type branched chain polypeptides

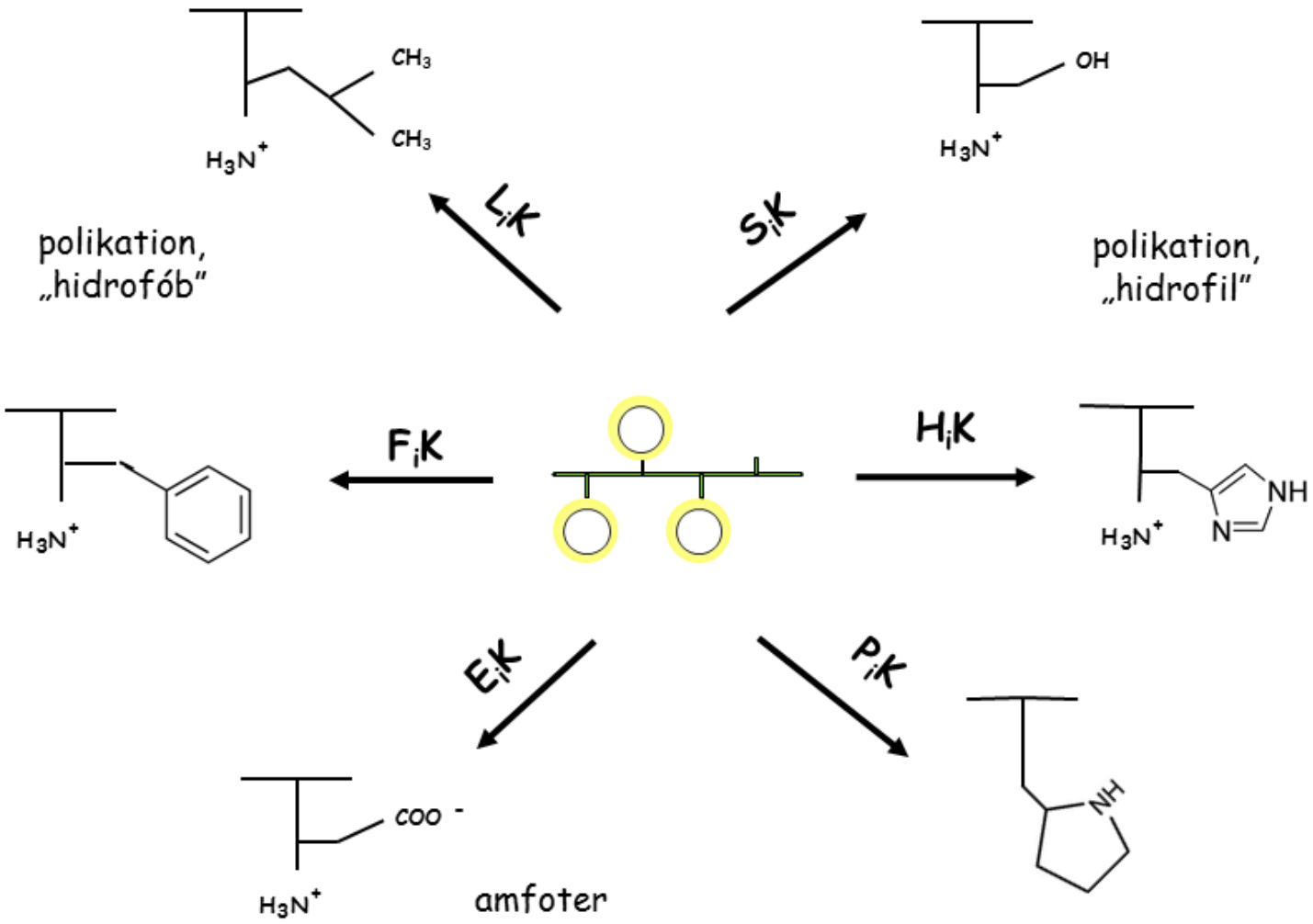


Preparation of XAK/XSK type branched chain polypeptides



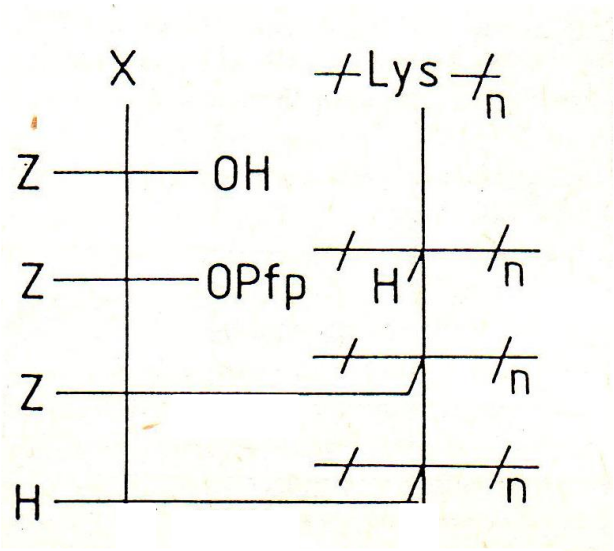


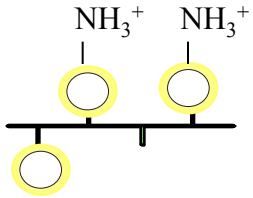
X_iK type branched chain polypeptides

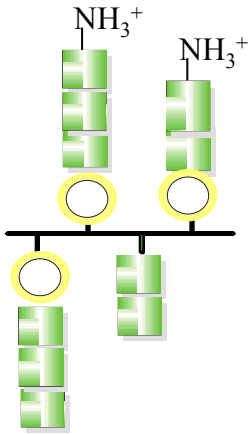


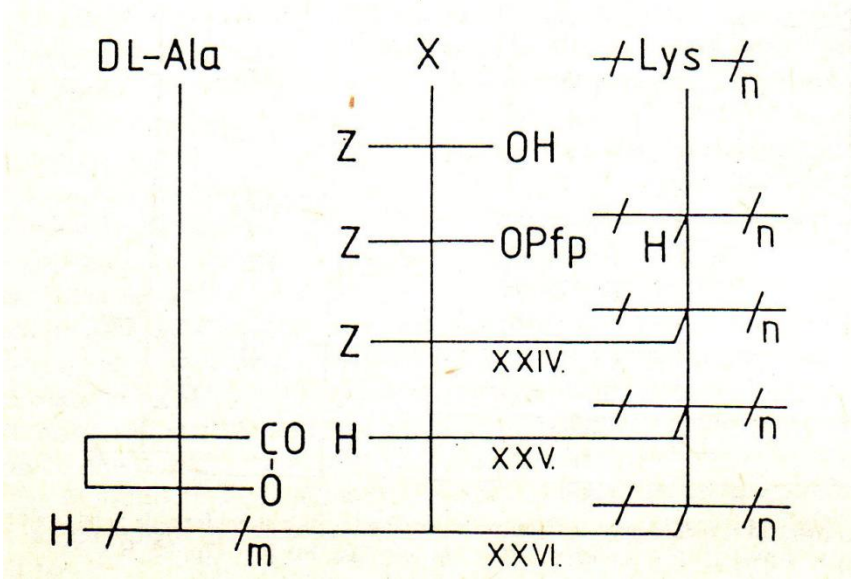
X = (D)Glu, (D)Leu, (D)Phe, (D)Ala, Ile, Pro, His or Ser.

Preparation of X_iK and AXK type branched chain polypeptides





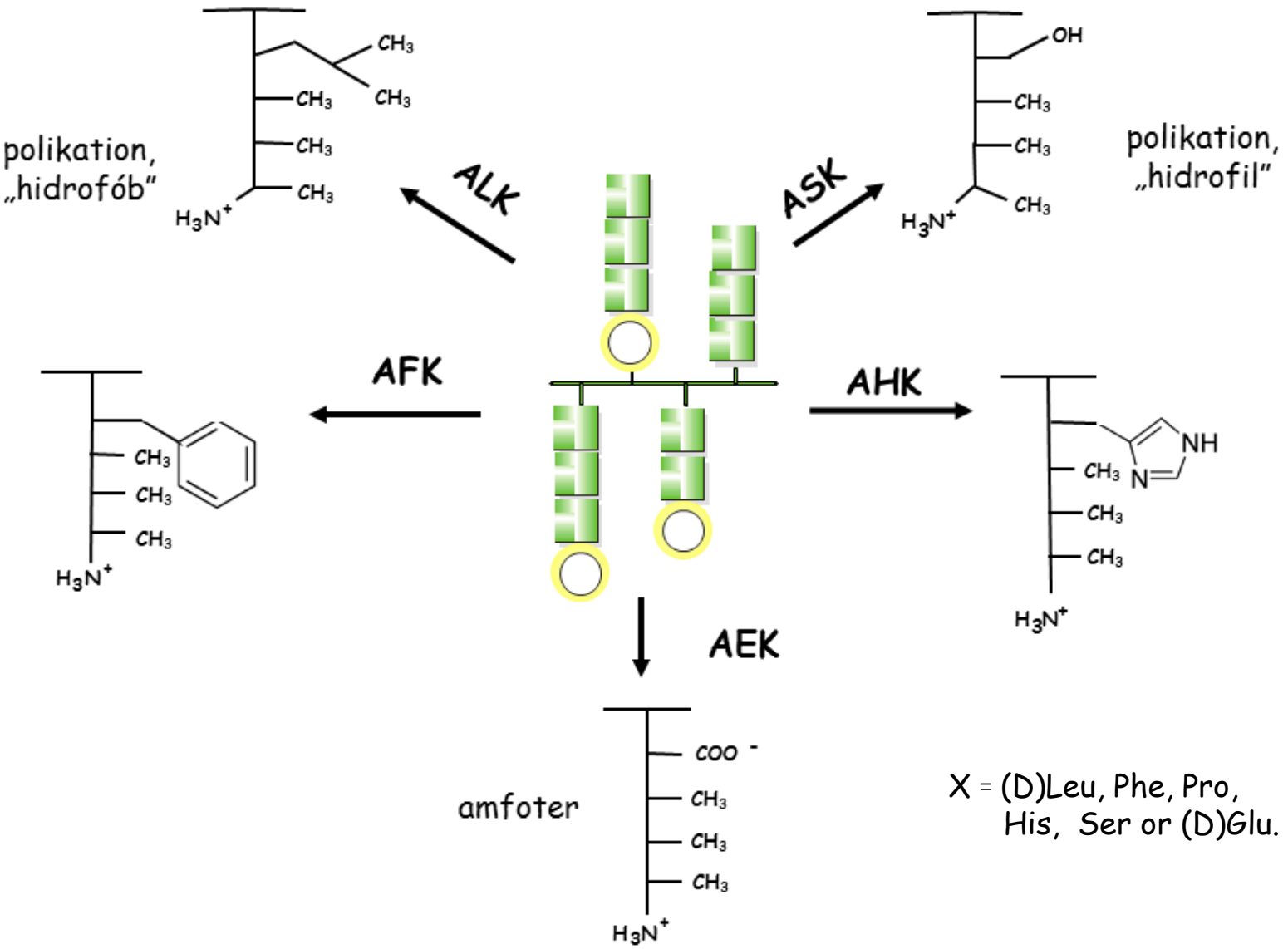




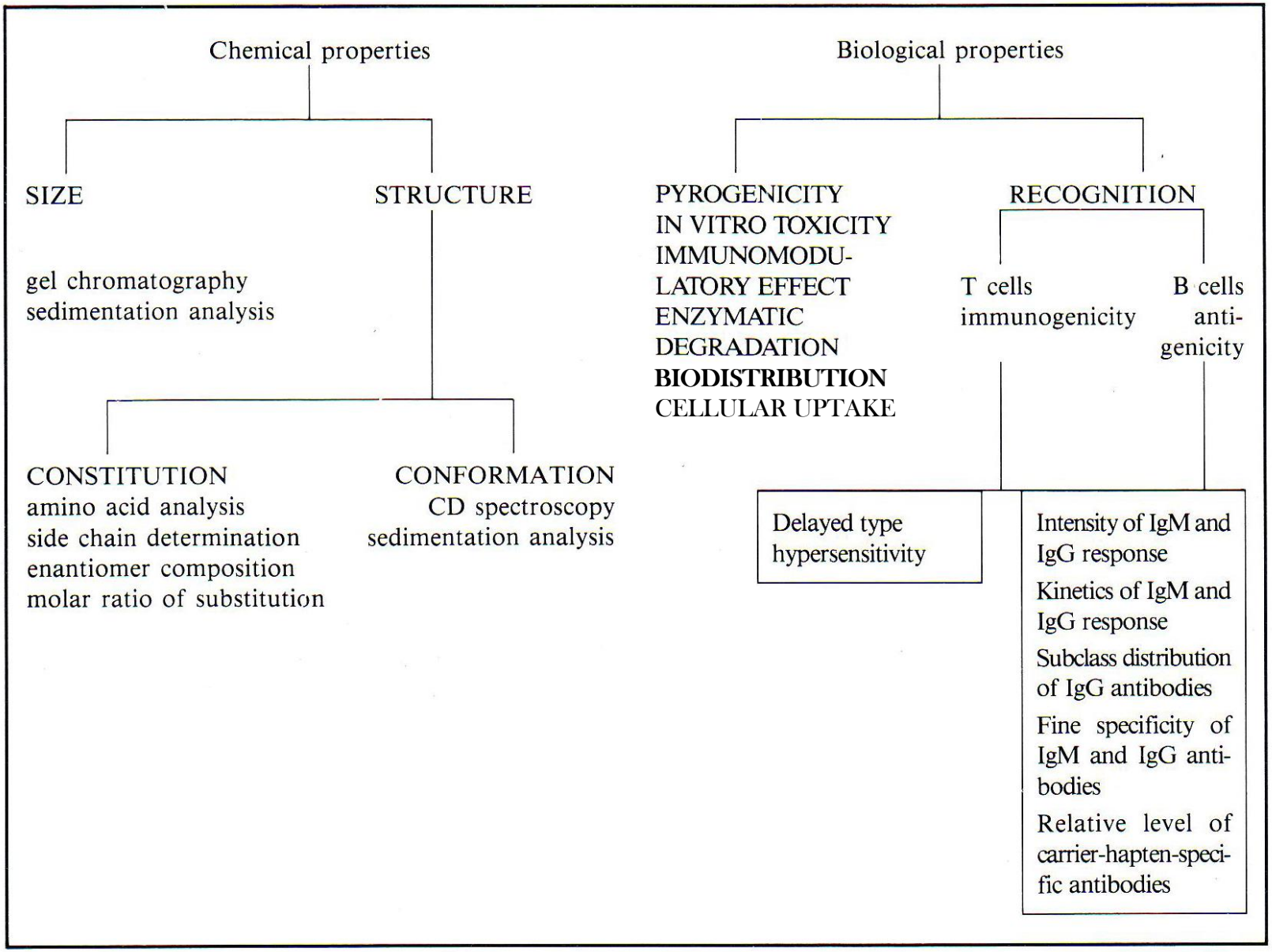
Collection Czechoslovak Chem. Commun. [Vol. 50] [1985]

Biopolymers, Vol. 28, 1801–1826 (1989)

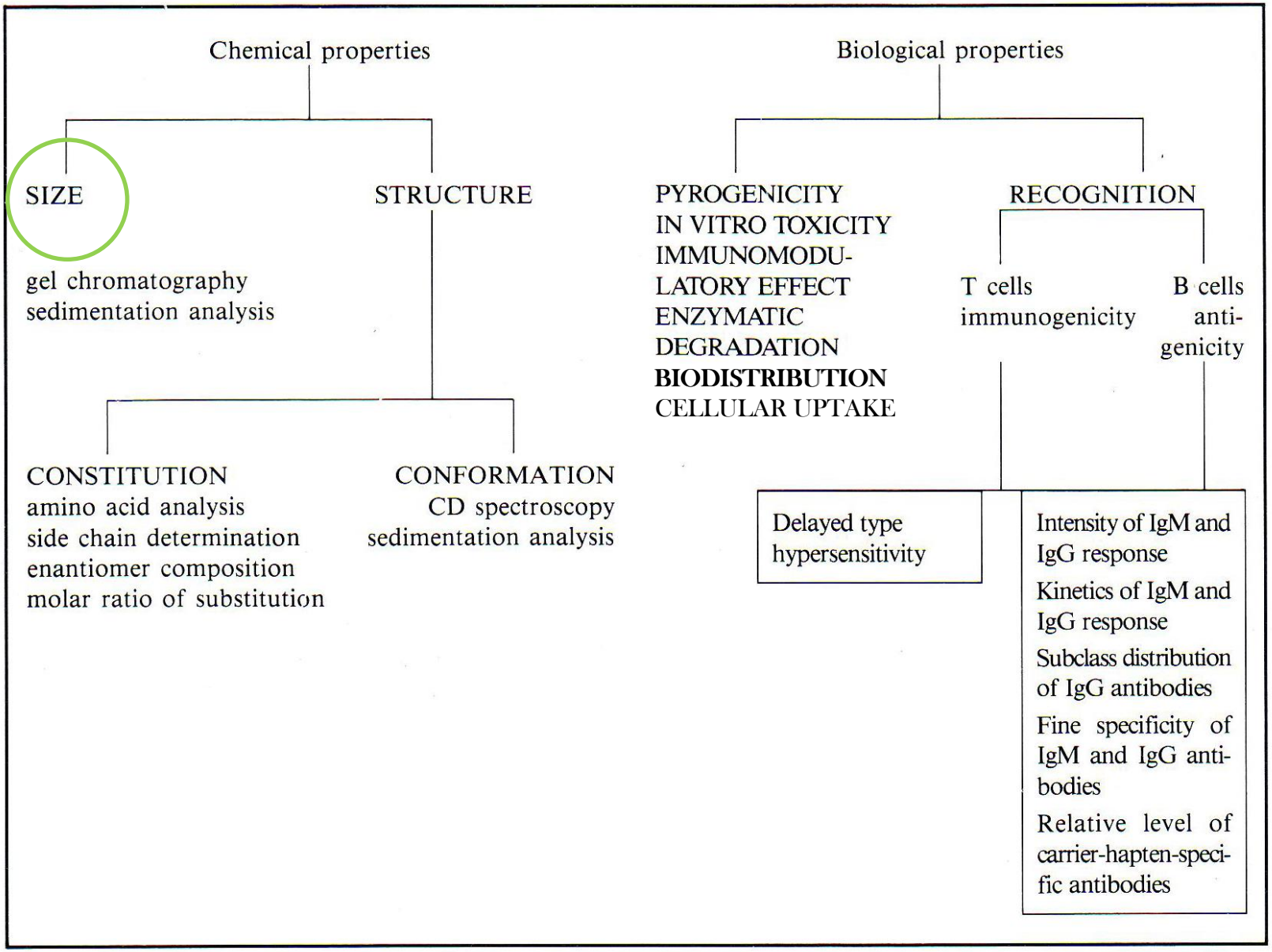
AXK type branched chain polypeptides



Characterization branched chain polypeptides



Characterization branched chain polypeptides



Colloid & Polymer Science

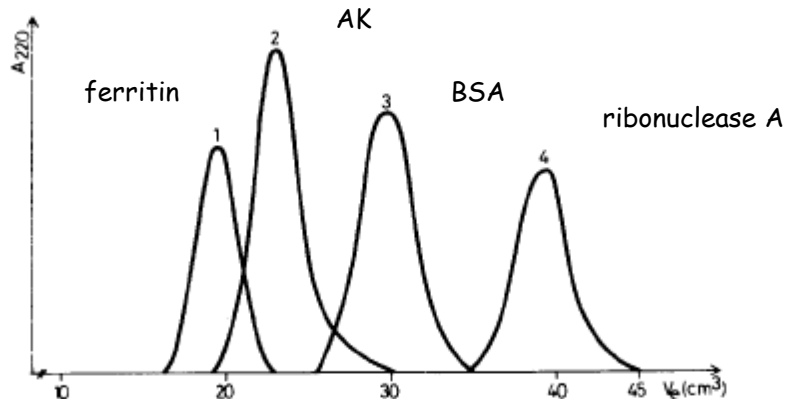
Colloid & Polymer Sci. **262**, 208–212 (1984)

GPC, CD and sedimentation analysis of poly-Lys and branched chain poly-Lys–poly-DL-Ala polypeptides*)

F. Hudecz, P. Kovács*), S. Kutassi-Kovács, and J. Kajtár**)

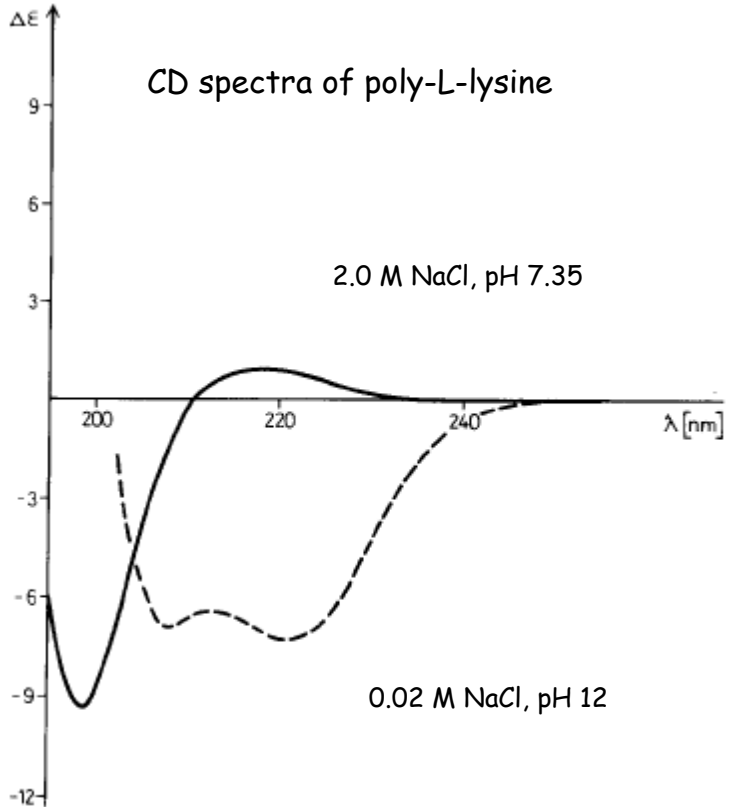
Table 1. Data on sedimentation analysis of poly-*L*-lysine samples and poly-*L*-Lys–poly-DL-Ala (AK)

	*p*Lys			AK
$\bar{M}_w$	30000	41000	94000	67000
$\bar{M}_z$	36000	45000	101000	77000
$\bar{M}_z/\bar{M}_w$	1.2	1.1	1.07	1.15
$\bar{M}_n$	25000	37000	88000	58000
$\overline{DP}_n$	120	180	430	174

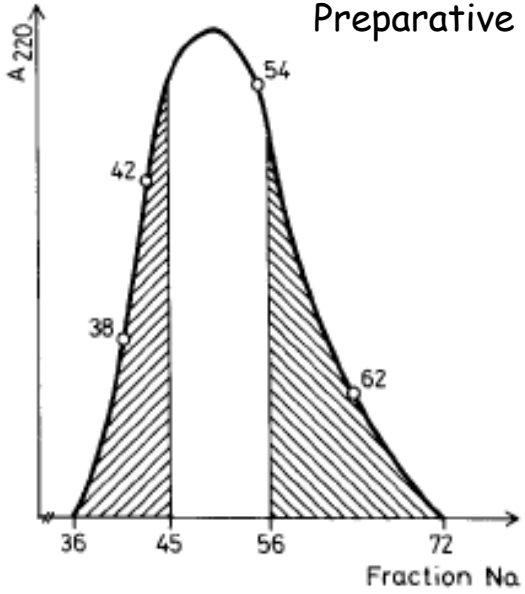


Sephadex *G*-200, 0.9 x 40 cm, 0.05 M $NaHCO_3$, 8 cm^3/h

CD spectra of poly-L-lysine

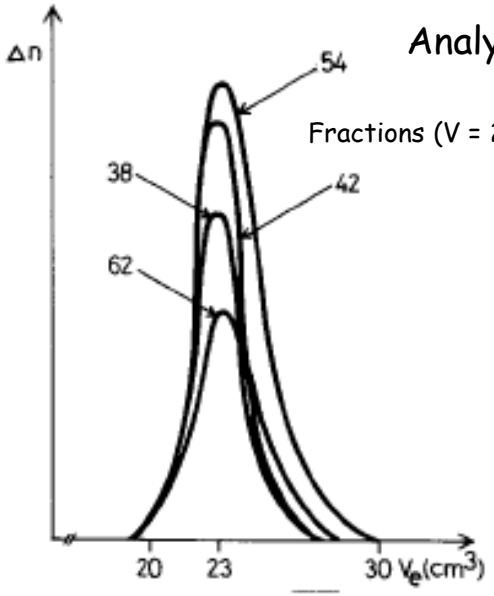


Preparative GPC – AK



Sephadex G-200,4 x 66 cm, 0.05 M $NaHCO_3$

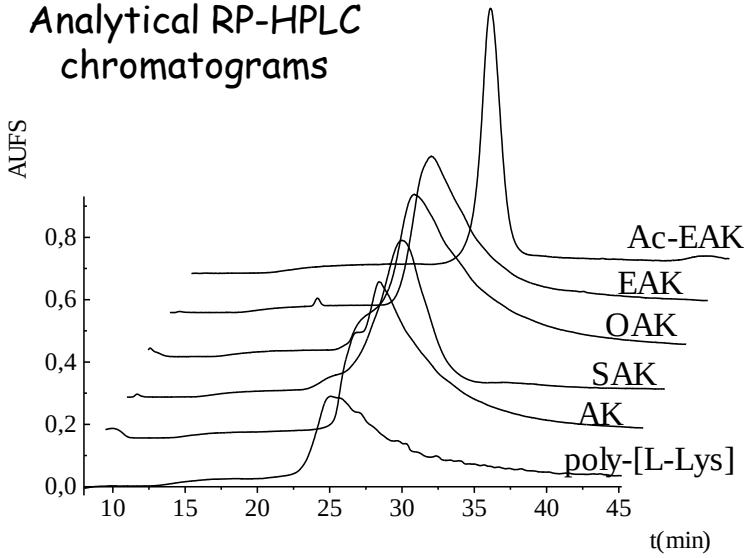
Analytical GPC – AK



Fractions (V = 2.5 cm^3) : 38, 42, 54 and 62

Sephadex G-200, 0.9 x 40 cm, 0.05 M $NaHCO_3$, 8 cm^3/h

Analytical RP-HPLC chromatograms



Waters (Milford, USA),
Delta Pak C_{18} column (300 x 3.9 mm I.D.)
with 15 mm silica (300 Å pore size)
eluent A: 0.1 % TFA
eluent B: 0.1% TFA in AcN-water (80:20, v/v %)
linear gradient 10% - 65% v/v of B in 45 min.
flow rate: 2 ml/min
UV detection at λ = 208 and 214 nm
polymer samples: 3mg/ml

Characterization branched chain polypeptides

Chemical properties

SIZE

gel chromatography
sedimentation analysis

STRUCTURE

CONSTITUTION
amino acid analysis
side chain determination
enantiomer composition
molar ratio of substitution

CONFORMATION
CD spectroscopy
sedimentation analysis

Biological properties

PYROGENICITY
IN VITRO TOXICITY
IMMUNOMODU-
LATORY EFFECT
ENZYMATIC
DEGRADATION
BIODISTRIBUTION
CELLULAR UPTAKE

RECOGNITION

T cells
immunogenicity

B cells
anti-
genicity

Delayed type
hypersensitivity

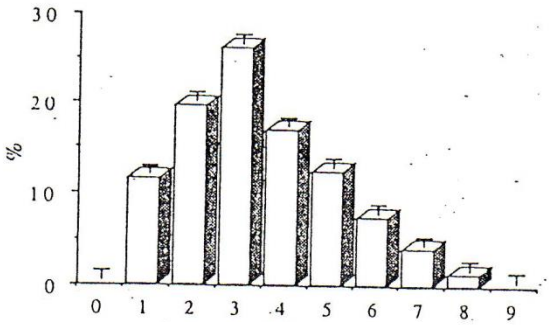
Intensity of IgM and IgG response

Kinetics of IgM and IgG response

Subclass distribution of IgG antibodies

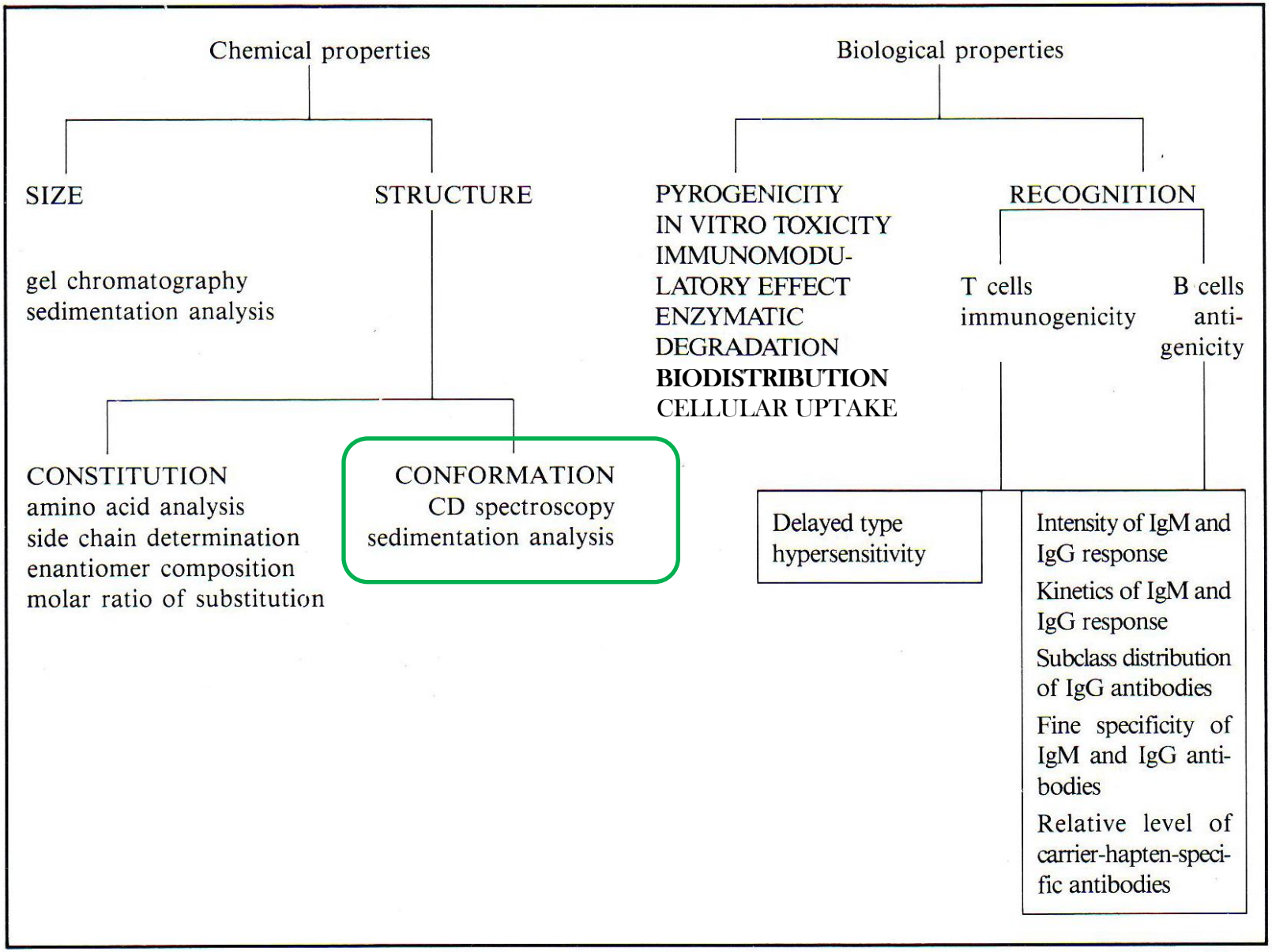
Fine specificity of IgM and IgG anti-bodies

Relative level of carrier-hapten-speci-fic antibodies



Length distribution of the oligo(DL-Ala) branches in AK

Characterization branched chain polypeptides



Primary structure - solution conformation

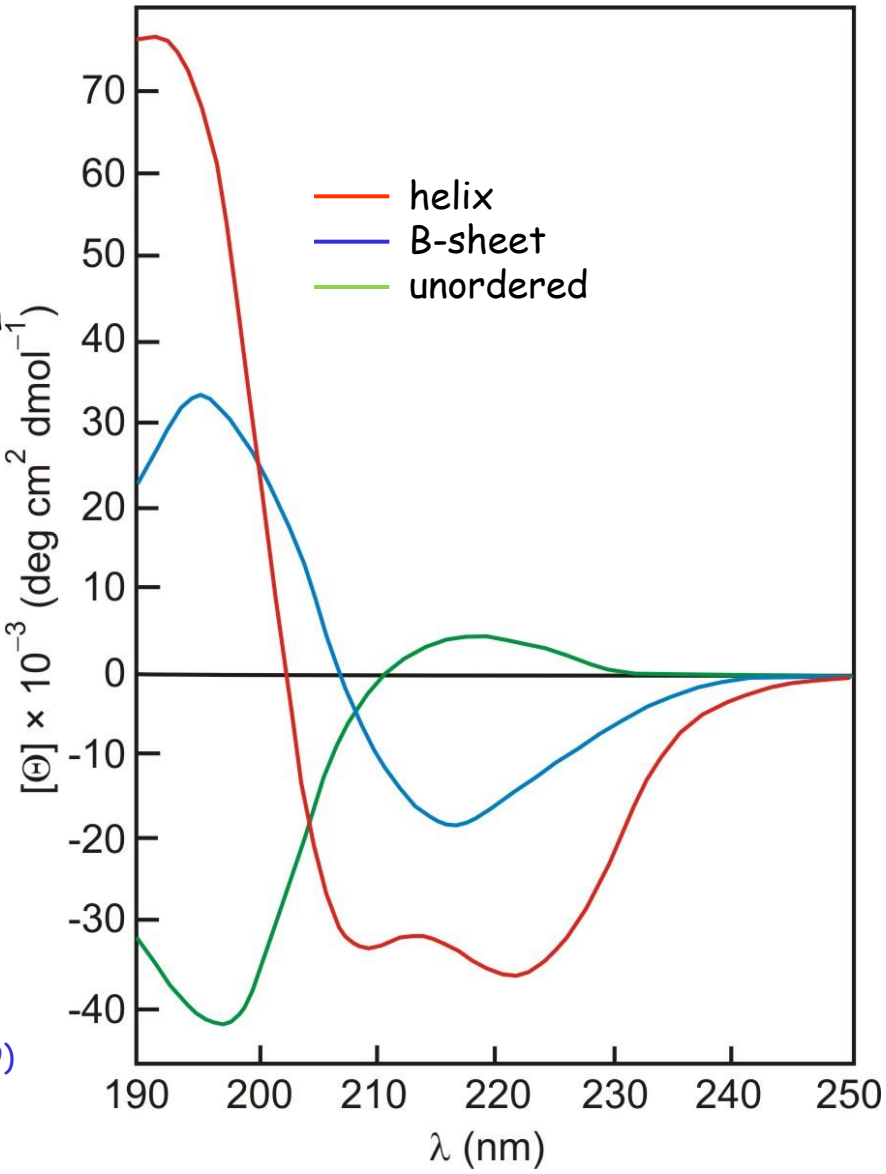
Amino acid X
- identity, character, charge
- position in the side the side chain
- number

could influence ordered structure formation

Method: ECD spectroscopy

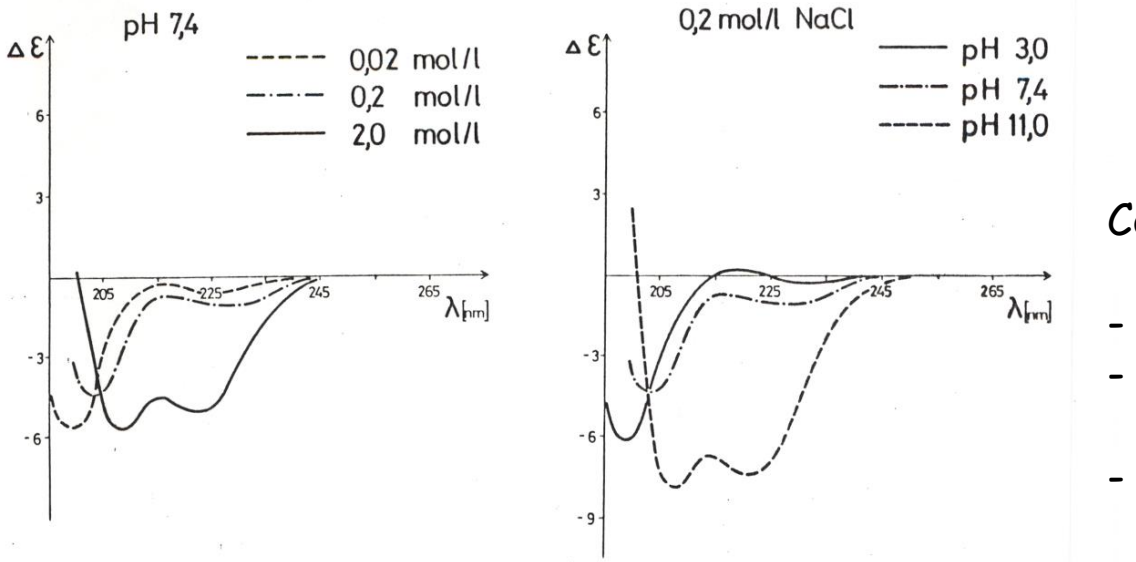
Conditions:

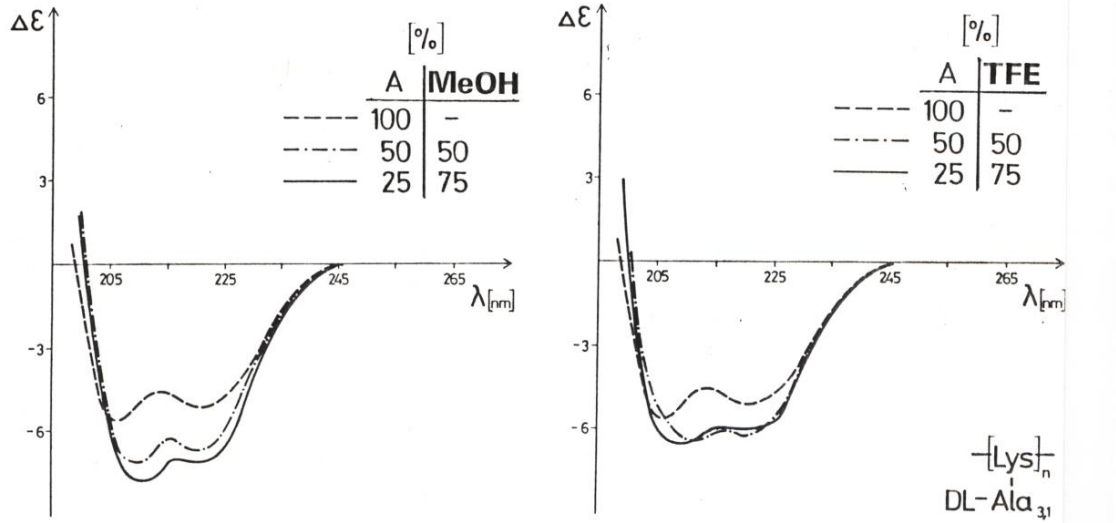
- pH: 3, 7.4 and 12
- Ionic strength:
  0.02, 0.2, 2.0 mol/dm^3 NaCl
- solvent:
  water, MeOH/water or
  TFE/water (25,50,75%)



N. Greenfield, G.D. Fasman: Biochemistry 8, 4108-4116 (1969)
N. Greenfield: Nature Protoc. 1, 2876-2880 (2006)

ECD spectrum – solution conformation



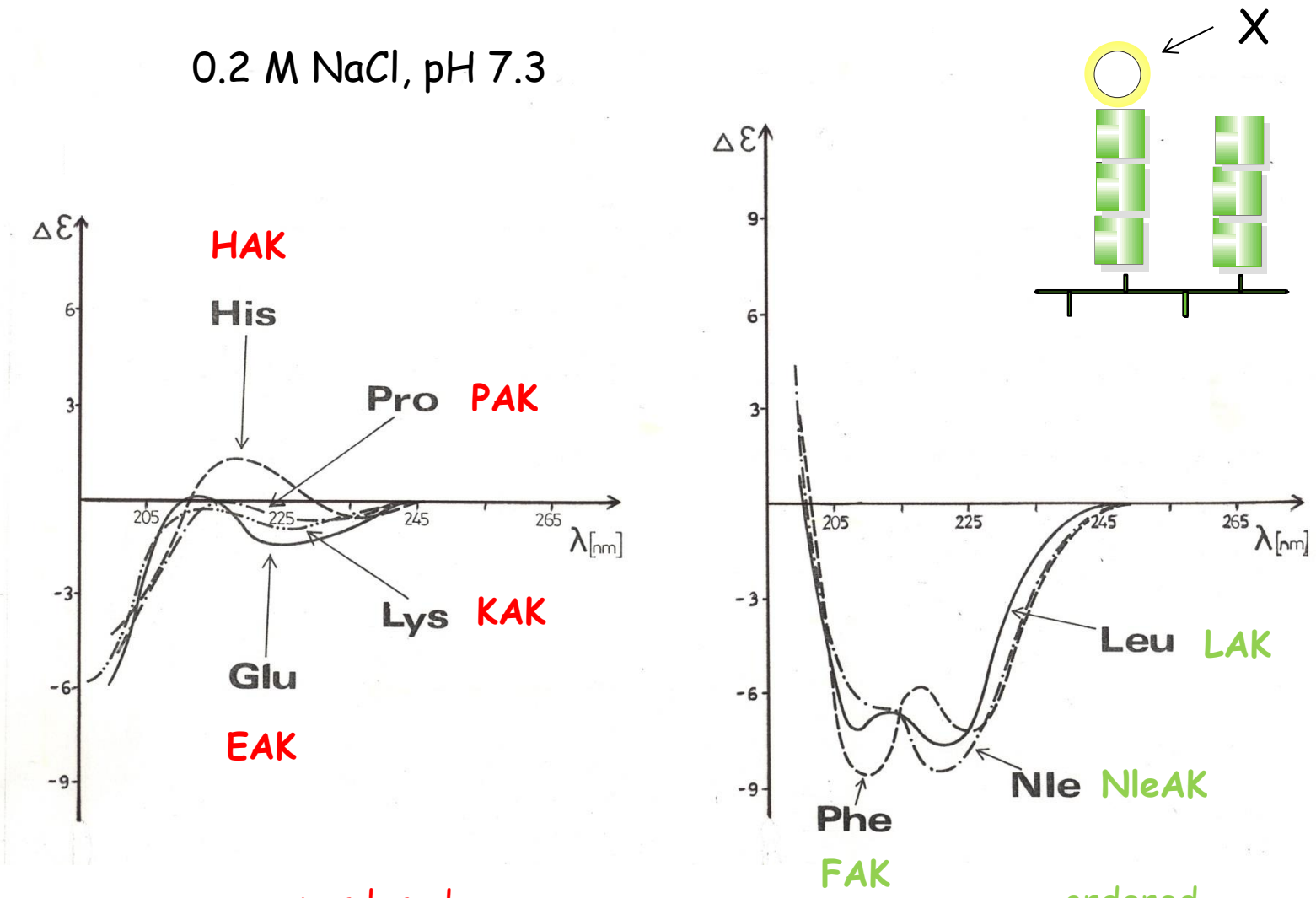


Conditions:

- pH: 3, 7.4 and 12
- ionic strength:
  0.02, 0.2, 2.0 mol/dm^3 NaCl
- solvent:
  water, MeOH/water or
  TFE/water (25,50,75%)

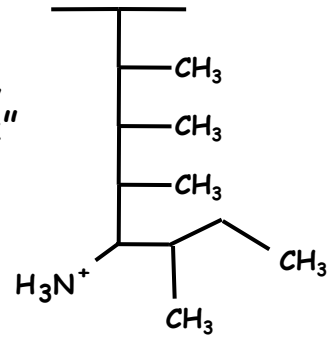
The effect of the identity of amino acid X

0.2 M NaCl, pH 7.3



The effect of the identity of amino acid X : Ile or Arg

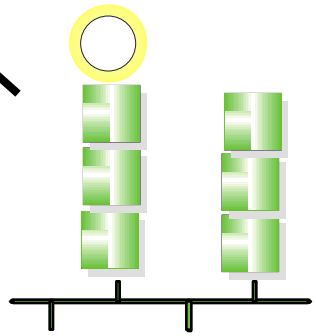
polycationic,
„hydrophobic"

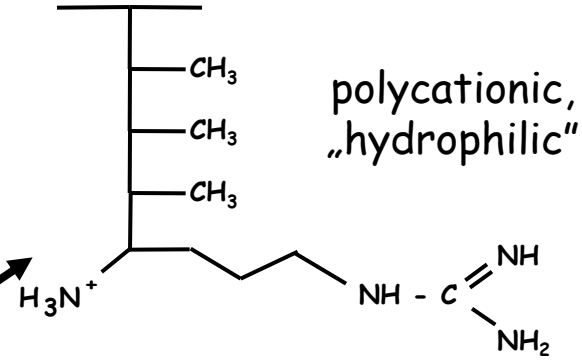


0.02 M NaCl

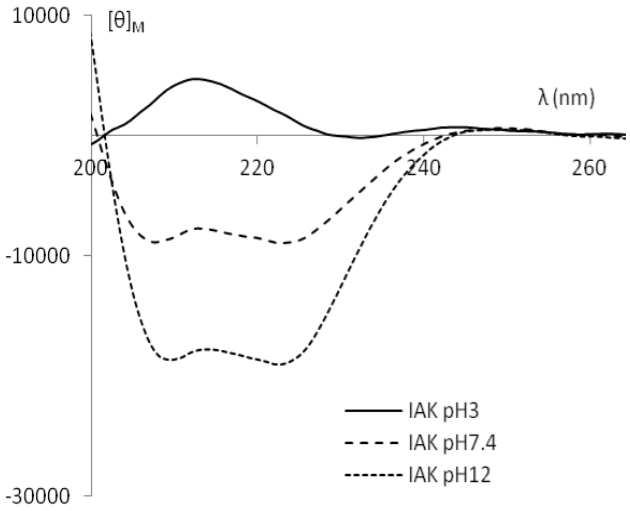
IAK

RAK

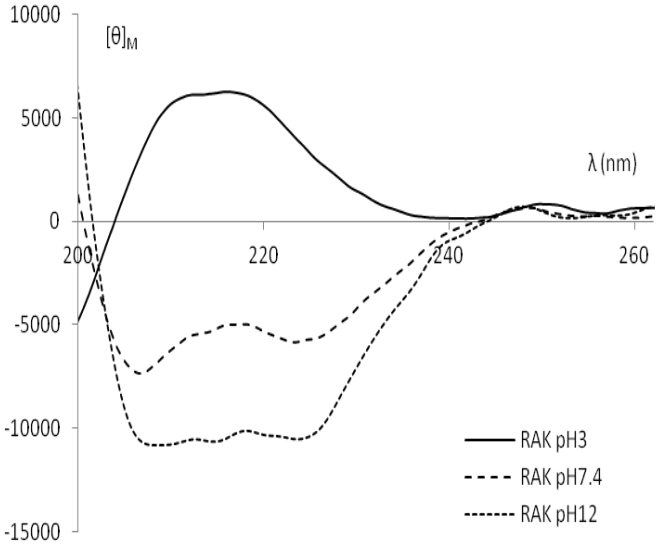




polycationic,
„hydrophilic"



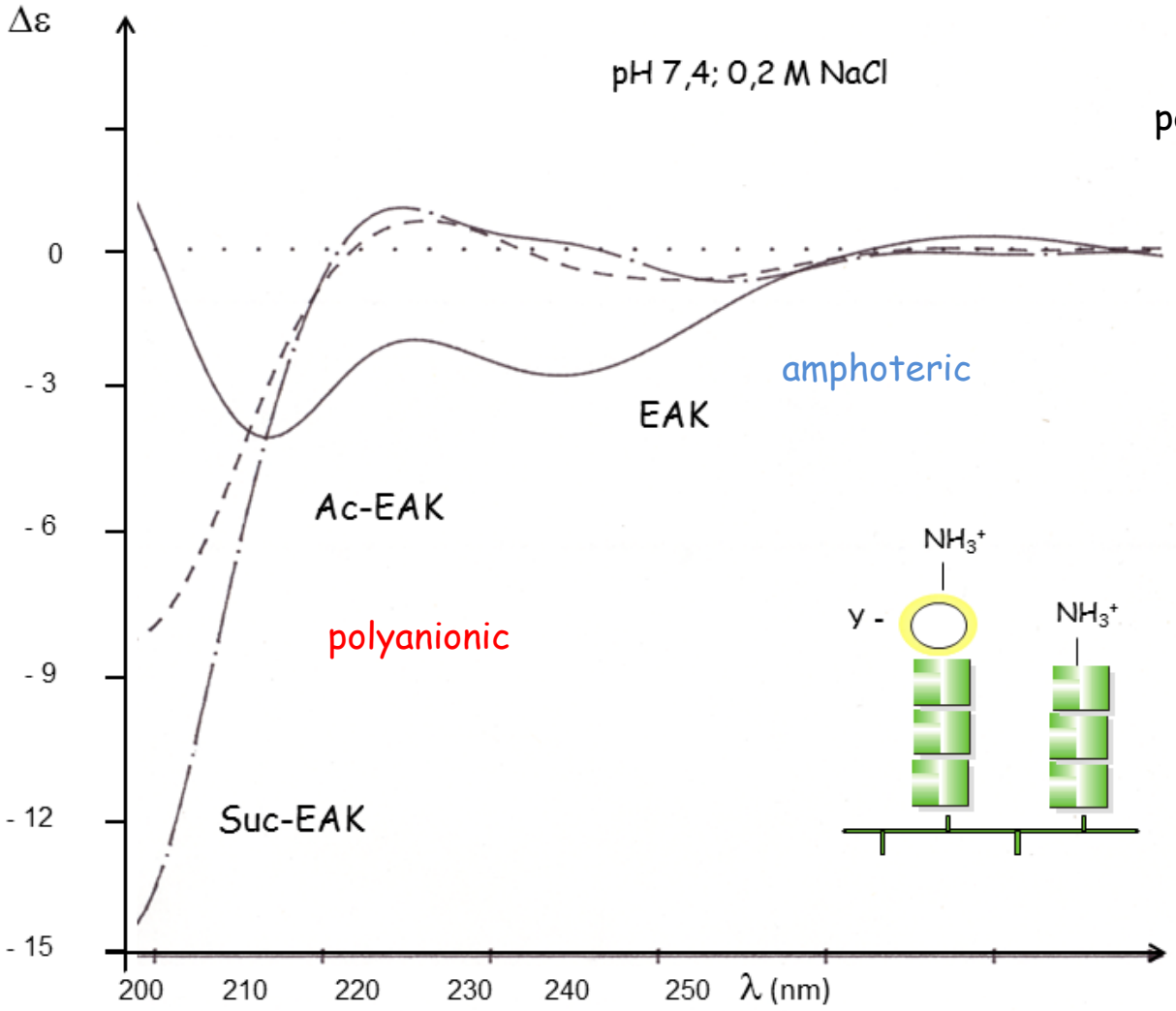
IAK, poli[Lys(Ilu$_i$-DL-Ala$_m$)]



RAK, poli[Lys(Arg$_i$-DL-Ala$_m$)]

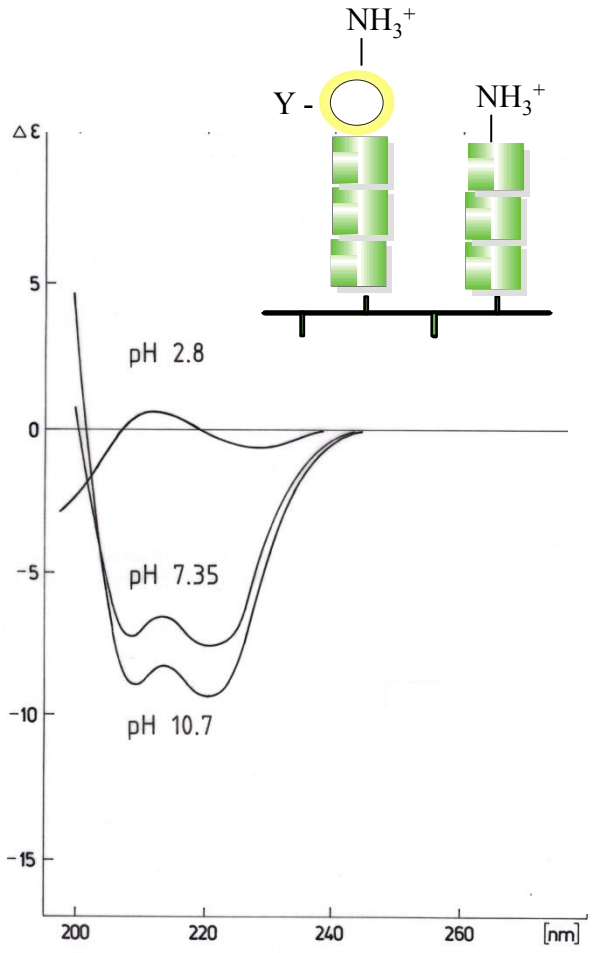
Sebestyén, M. et al. Structural Chemistry, 28: 527-536 (2017)

The effect of the charge of the terminal amino acid X : Glu (EAK), Ac-EAK or Suc-EAK

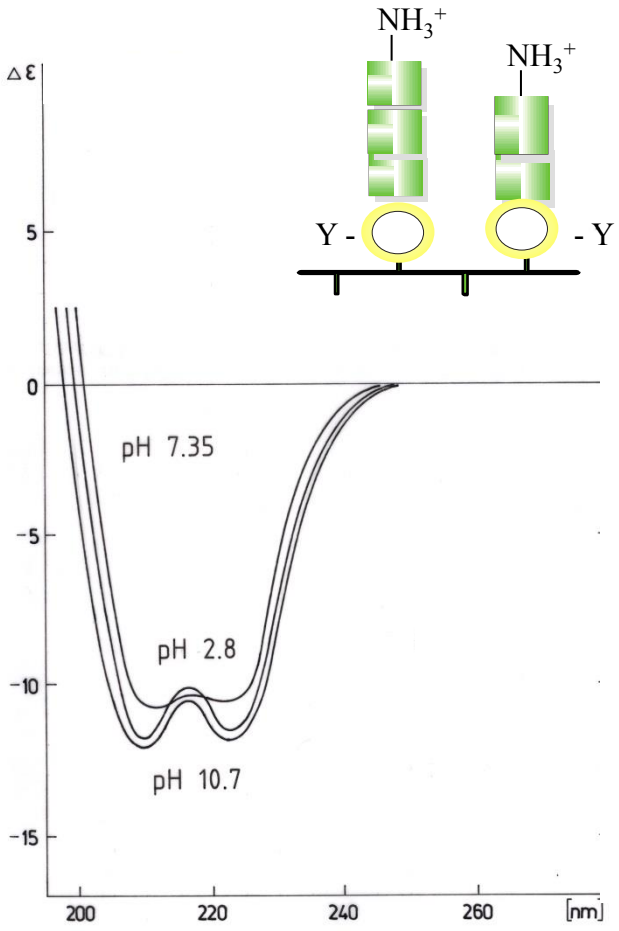


The effect of the position of hydrophobic amino acid X

0.2 M NaCl



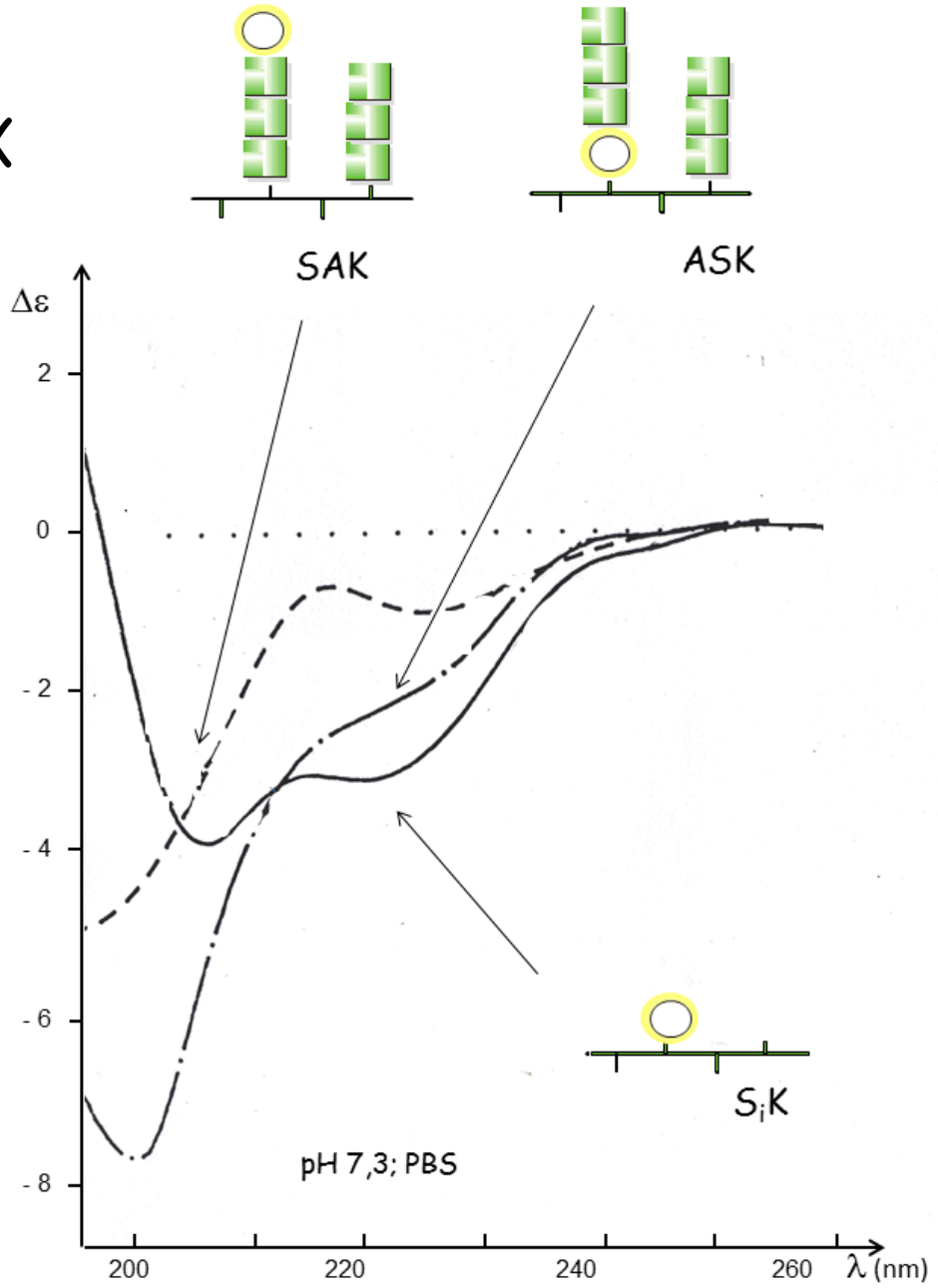
LAK, poly[Lys(Leu$_i$-DL-Ala$_m$)]



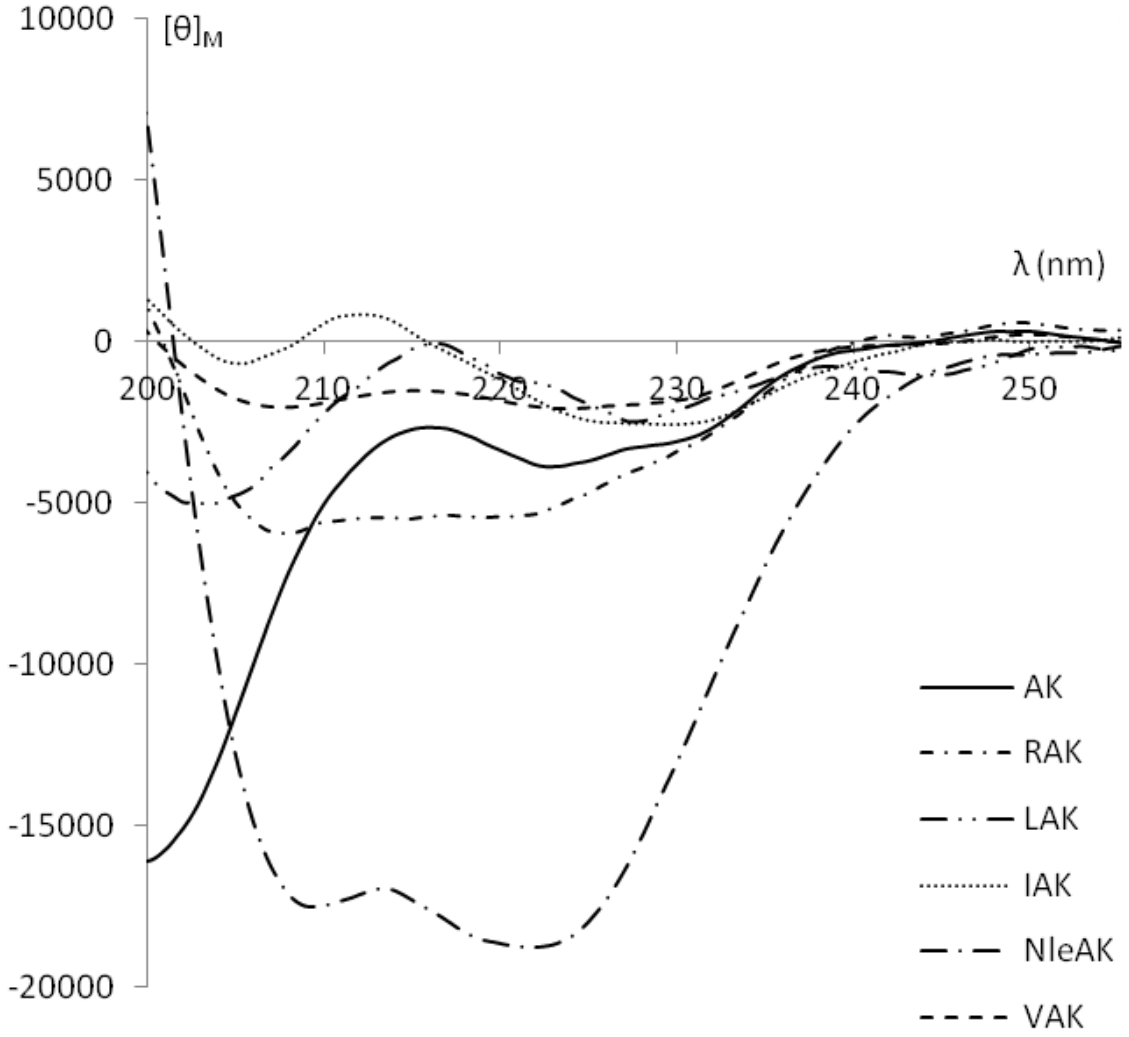
ALK, poly[Lys(DL-Ala$_m$-Leu$_i$)]

The effect of the position of hydrophilic amino acid X

X = Ser

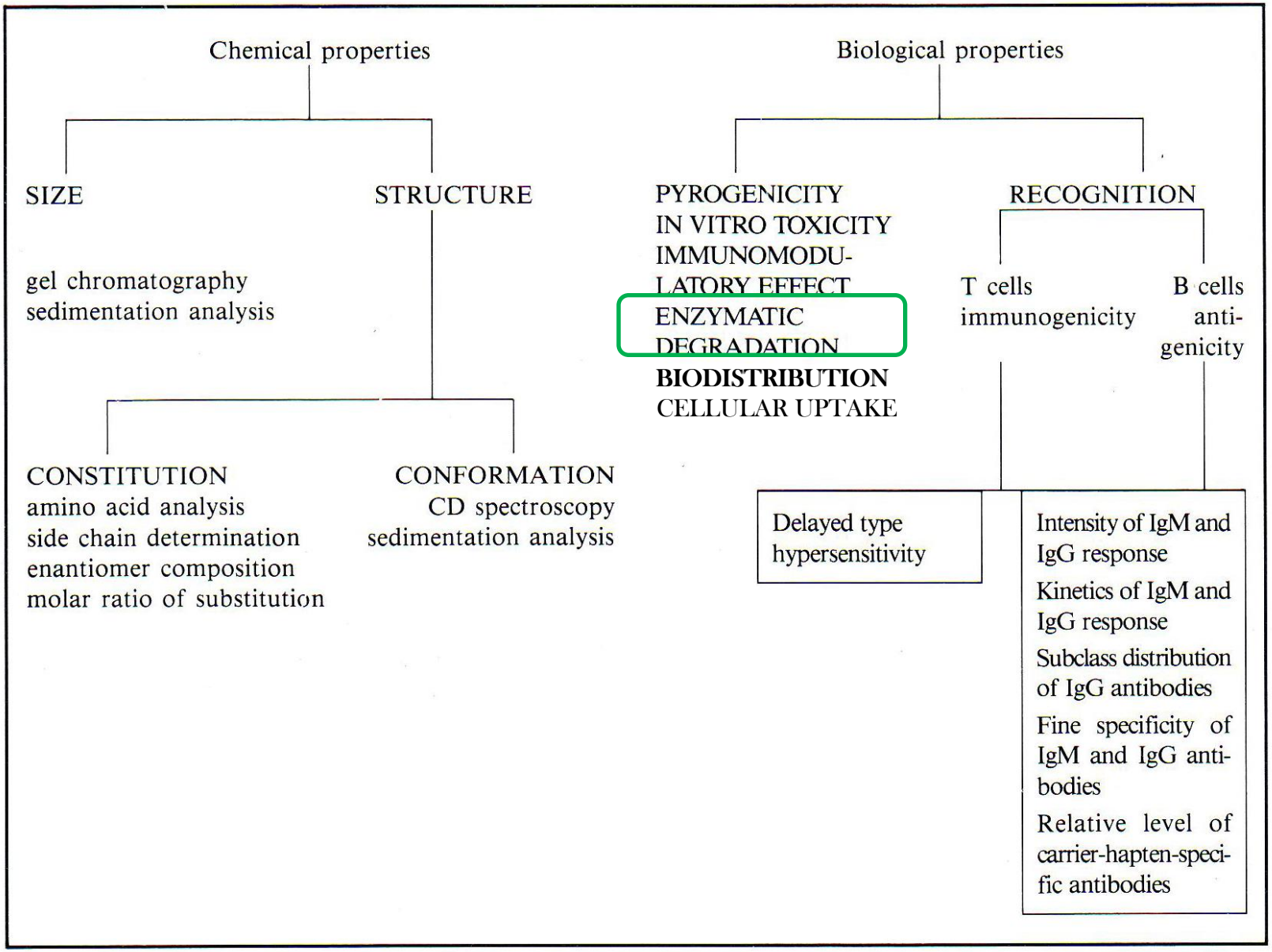


ECD spectra of branched polypeptides under nearly physiological conditions – a summary

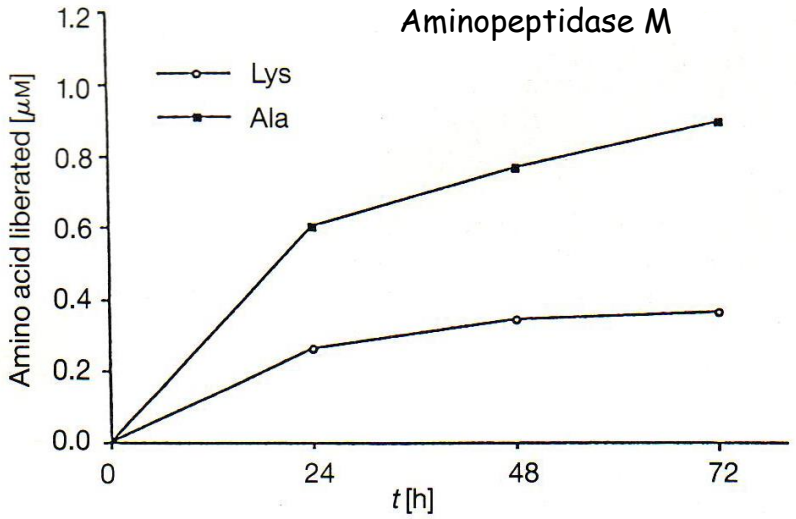


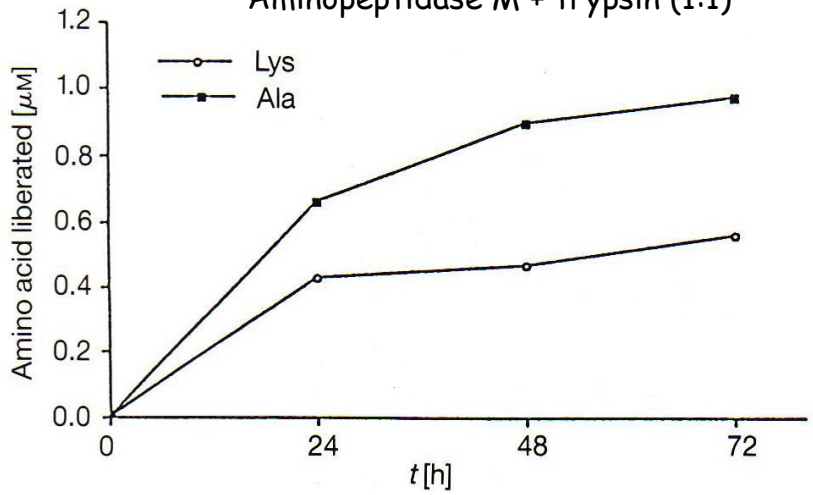
0.2 M NaCl
pH ~7.4
room temperature

Characterization branched chain polypeptides

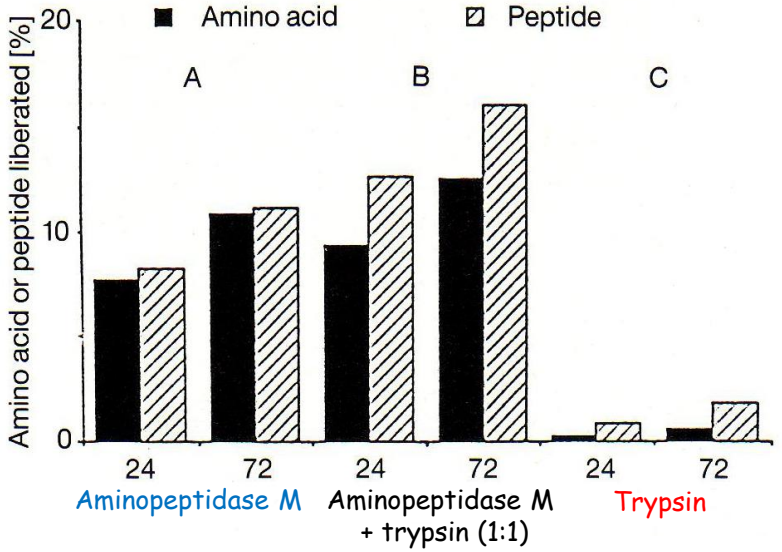


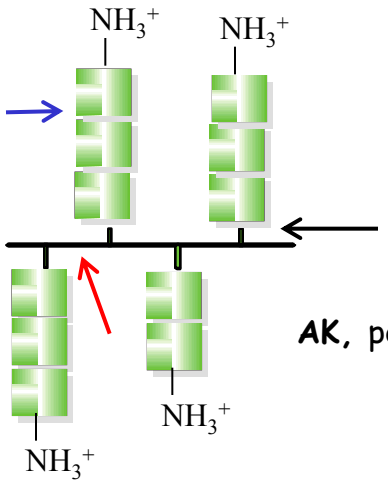
Branched polypeptides - biodegradation





0.02 M phosphate, pH 8

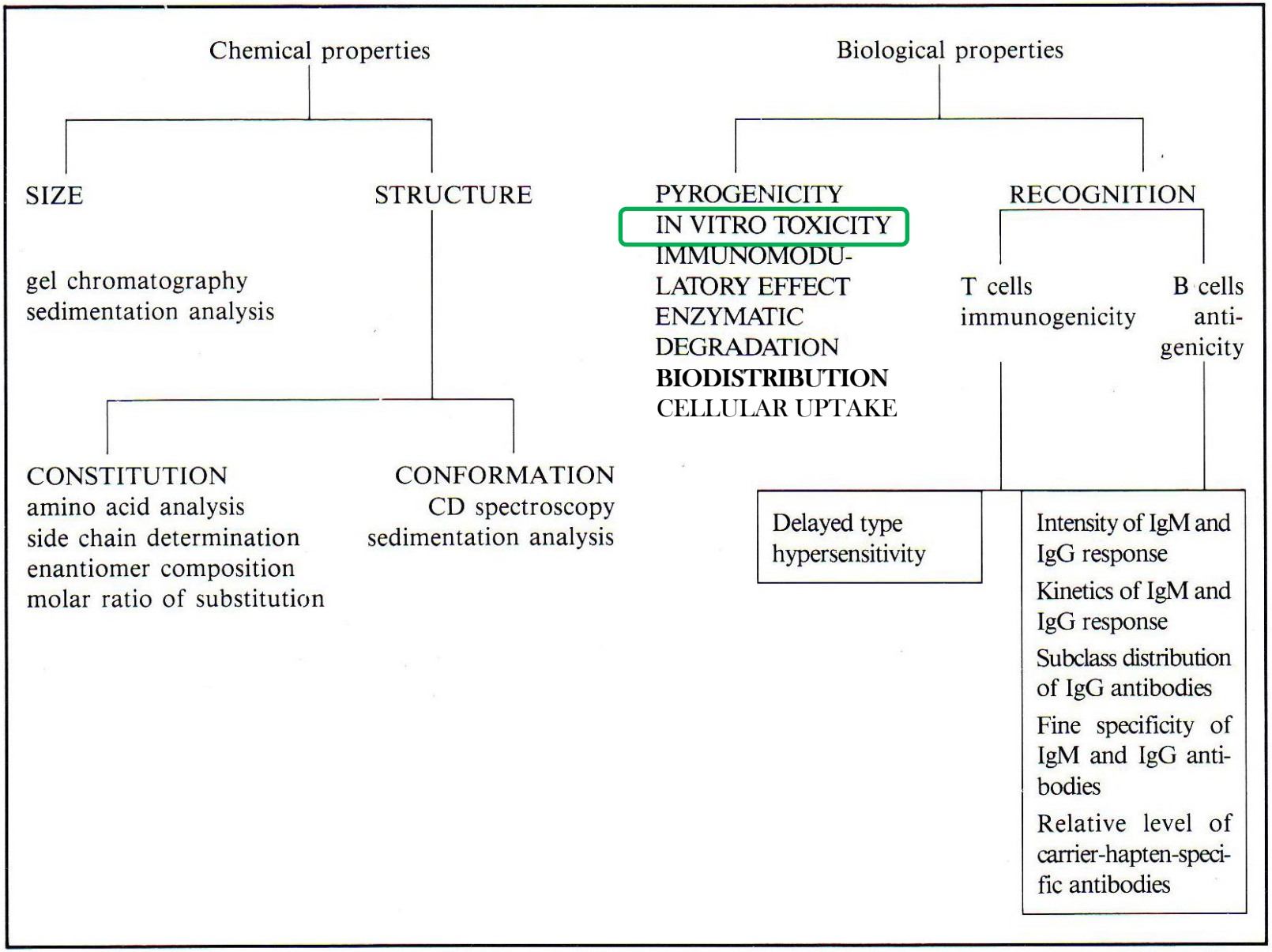




AK, poly[Lys(DL-Ala_m)]

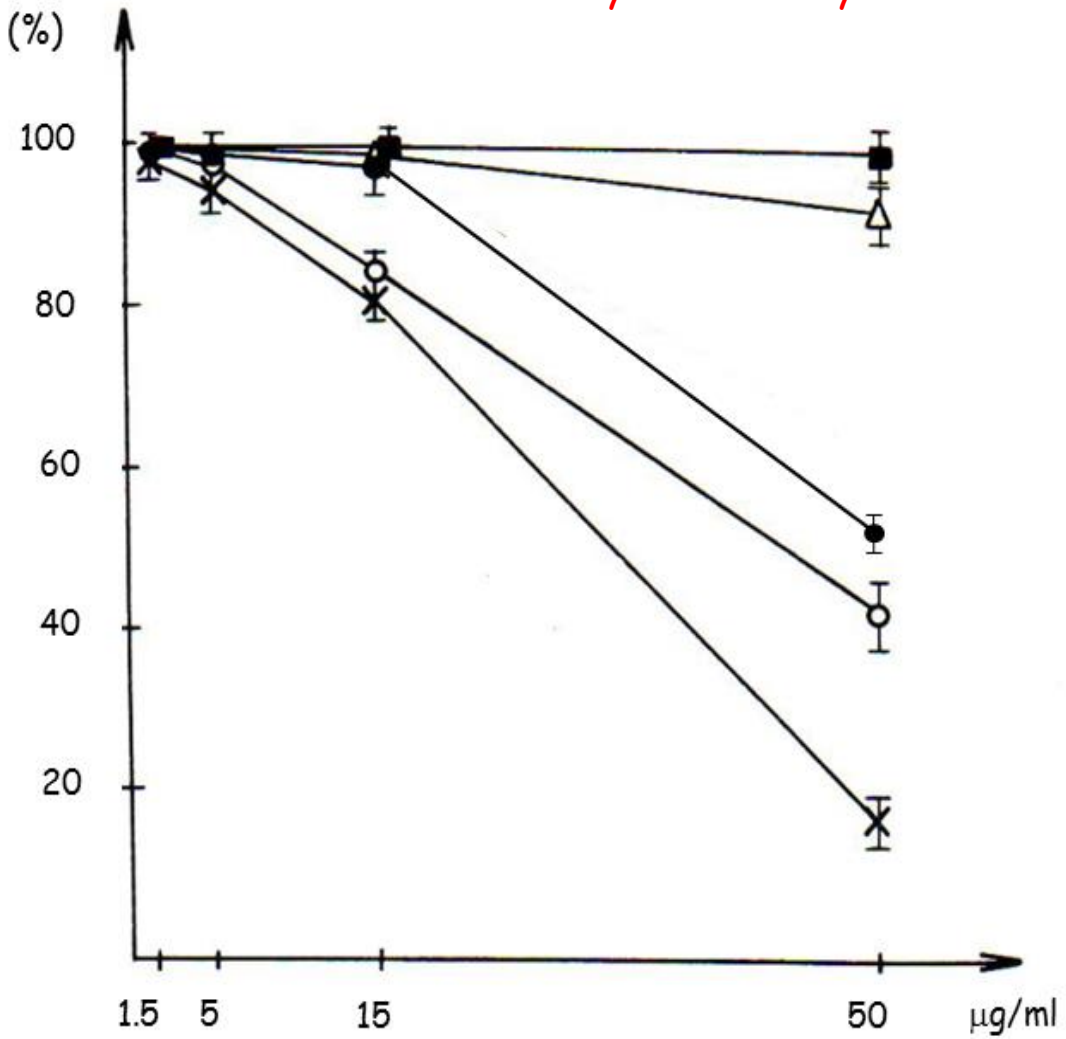
Biol. Chem. Hoppe-Seyler
Vol. 370, pp. 1019–1026, September 1989

Characterization branched chain polypeptides



The effect of position and identity of amino acid

in vitro cytotoxicitity on isolated rat liver cells

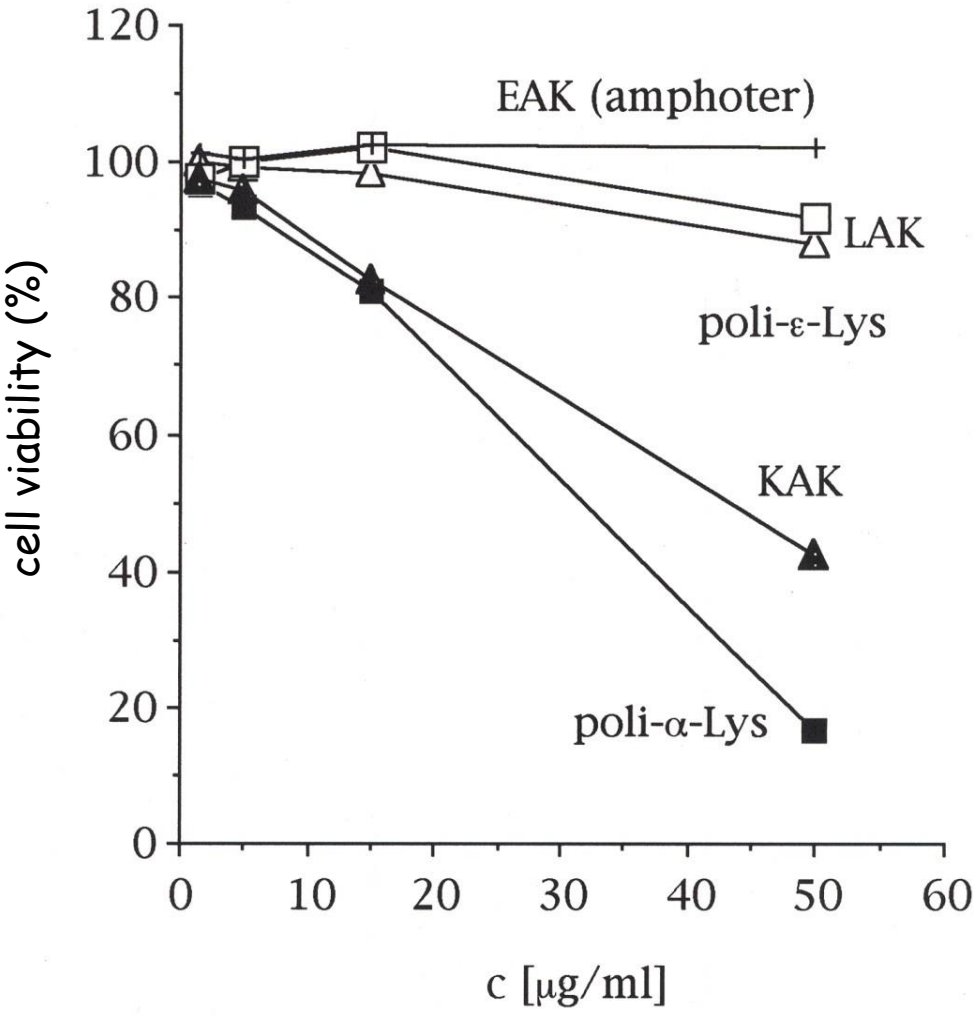


■ = EAK, AEK, SAK, ASK
Δ = LAK, ALK, D-LAK
FAK, D-FAK
HAK, D-HAK, PAK,
D-EAK
poly[Lys(Lys)]
● = AK (m = 3)
○ = KAK, D-KAK
× = poly[Lys]

Incubation: 1 hr, 37° C

The effect of charge - the identity of amino acid X:

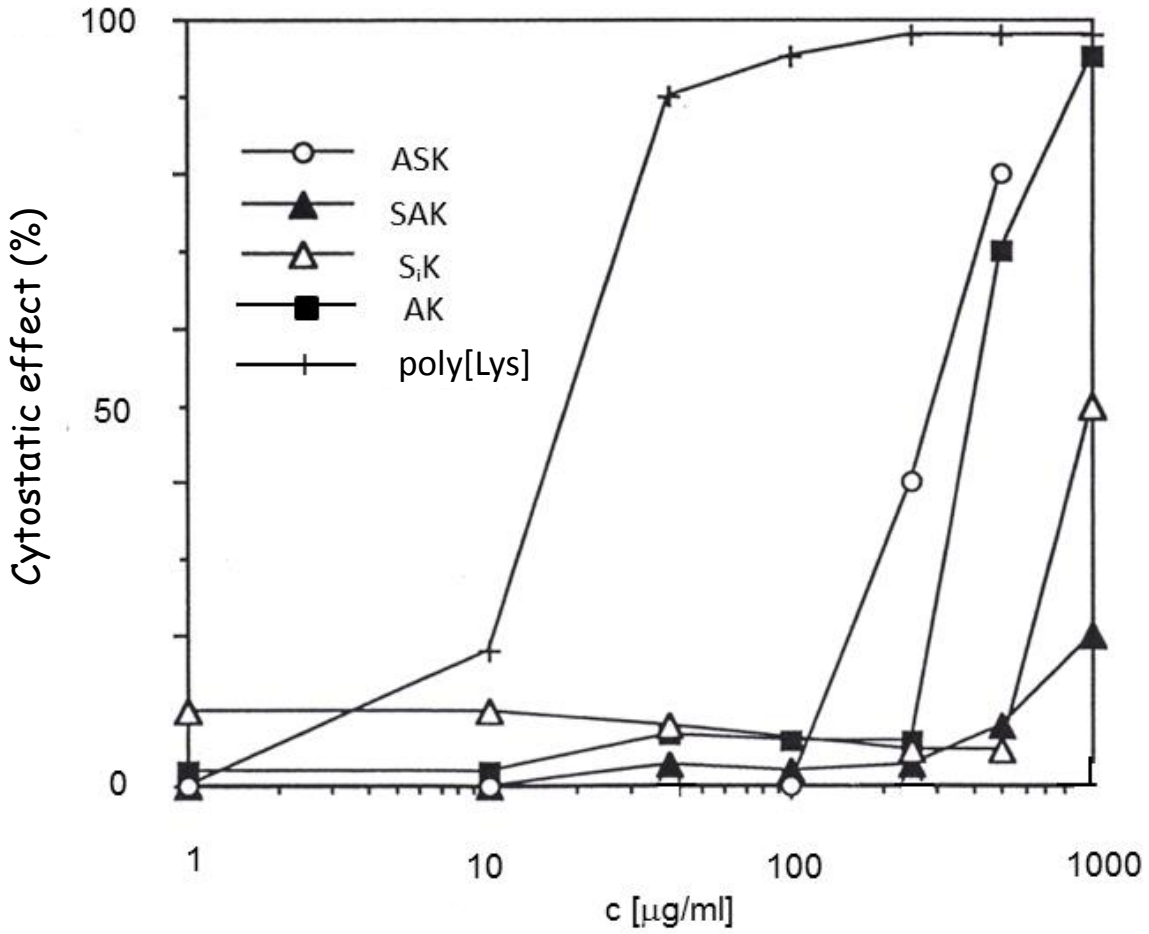
in vitro cytotoxicitity HeLa cells



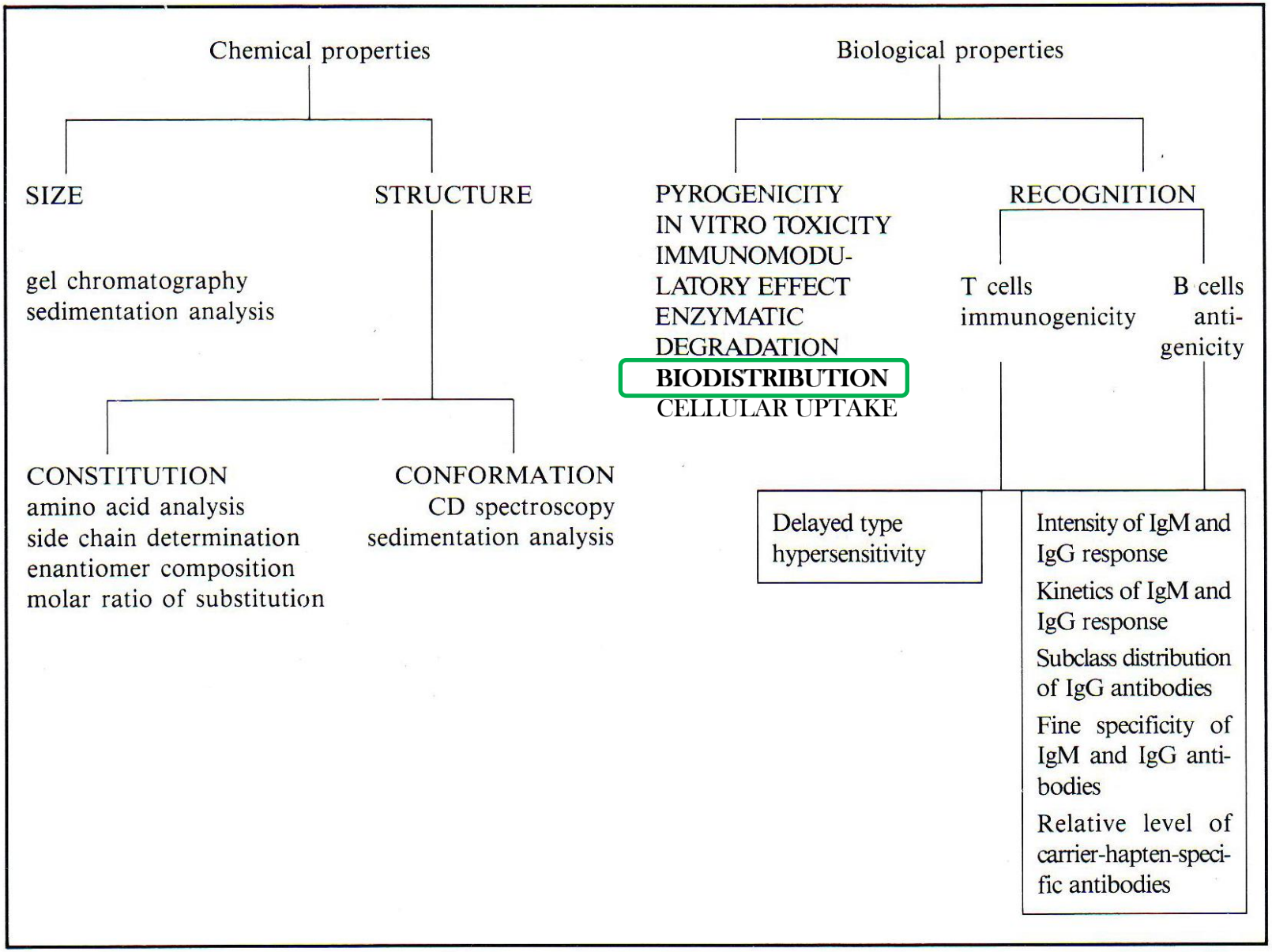
The effect of the position of hydrophilic amino acid (X = Ser) on *in vitro* cytostatic activity

C26 mouse carcinom cells

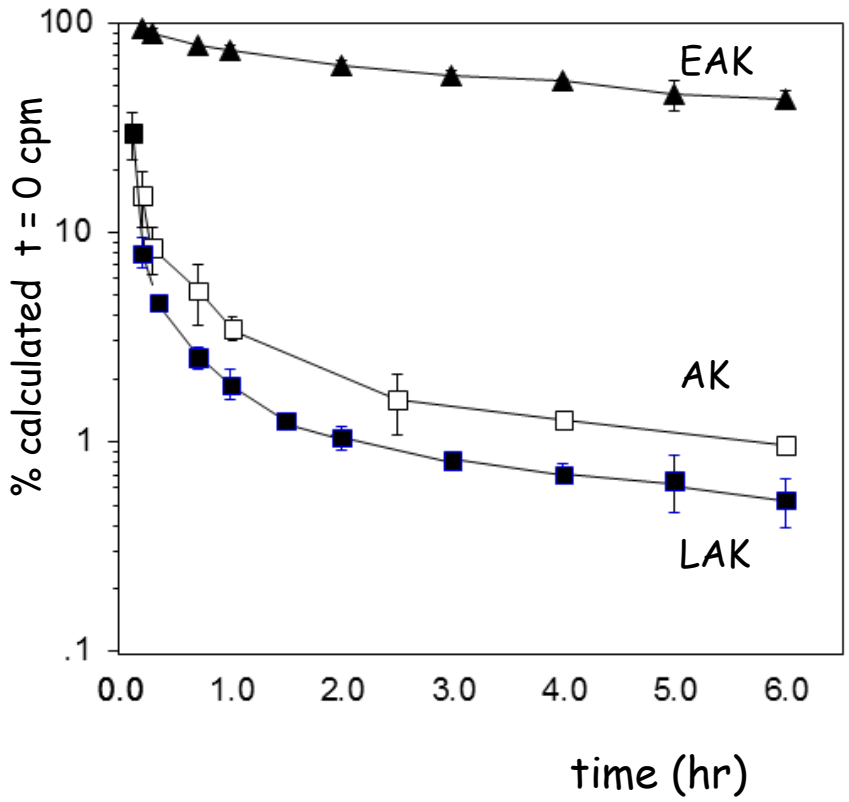
Incubation: 3 hrs, 37° C

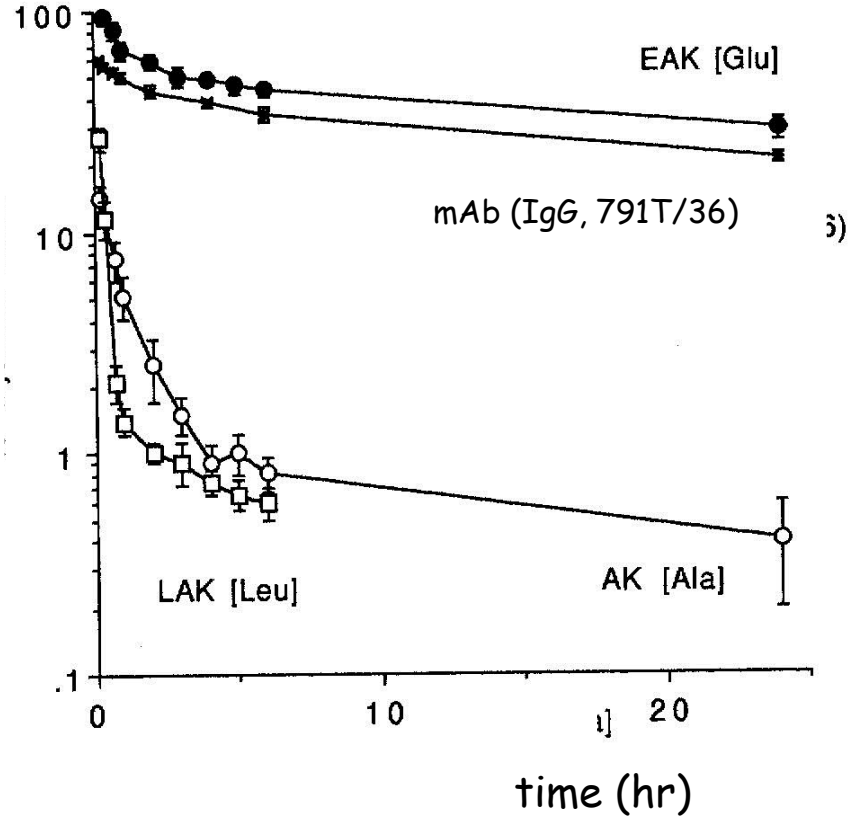


Characterization branched chain polypeptides



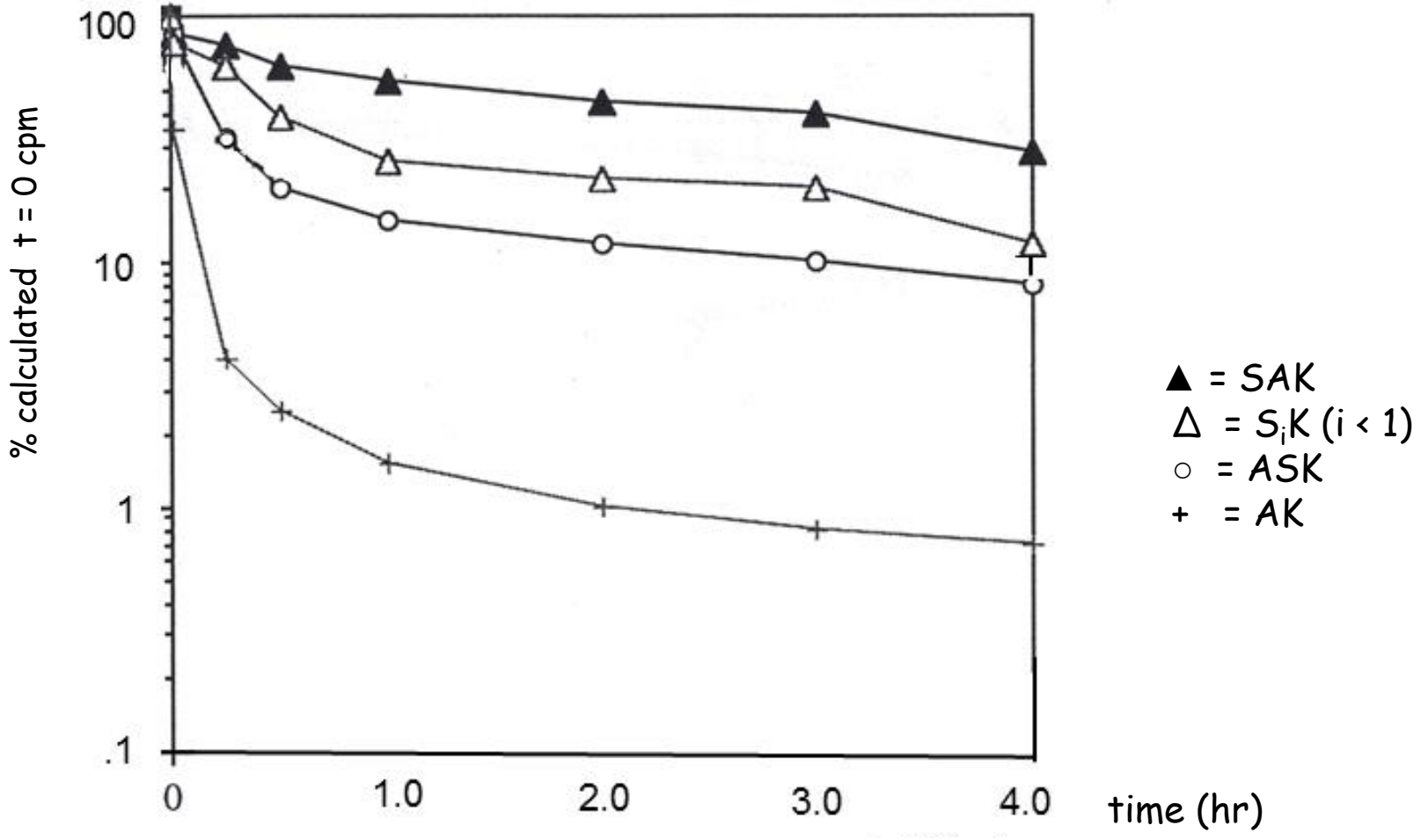
Blood clearance: the effect of the identity of amino acid X comparison with mAb





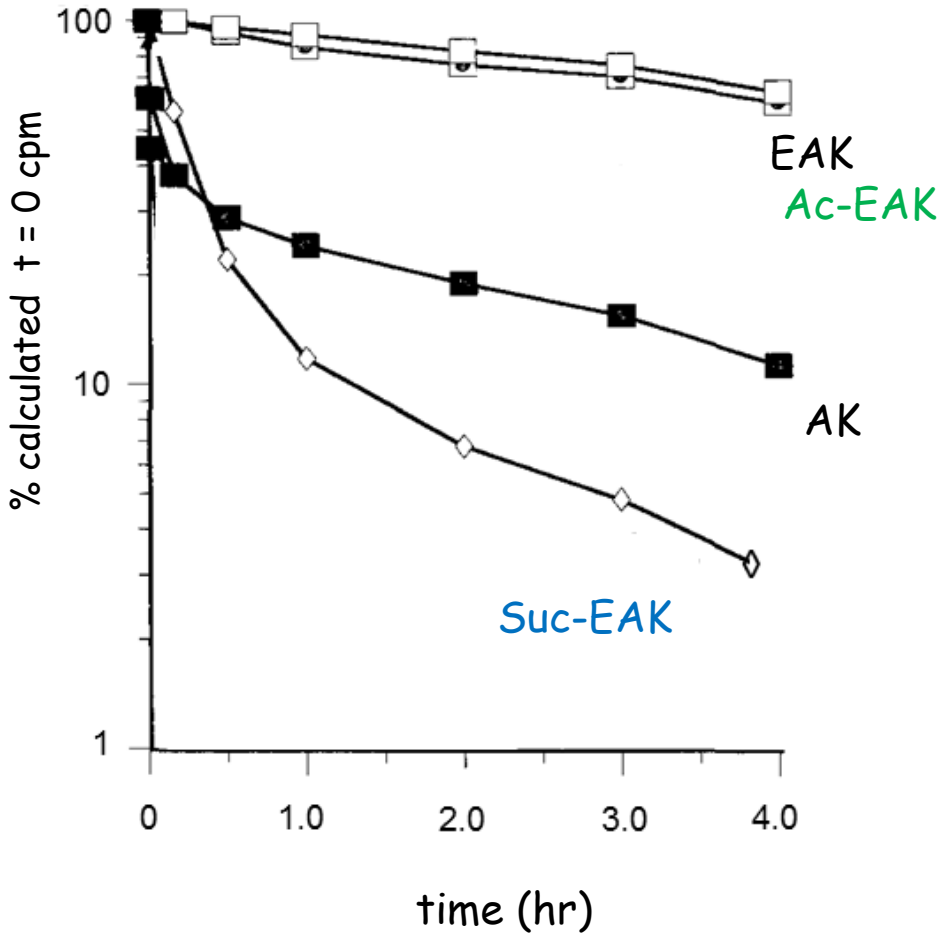
I^{125} isotope labelled polypeptides/protein i.v. injection

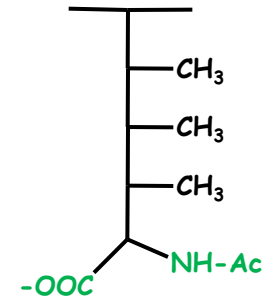
The effect of the position of hydrophilic amino acid (X = Ser) on blood clearance in Balb/c mice

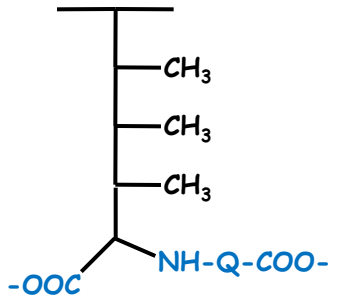


I^{125} isotope labelled polypeptides i.v. injection

The effect of the position of hydrophilic amino acid X on blood clearance



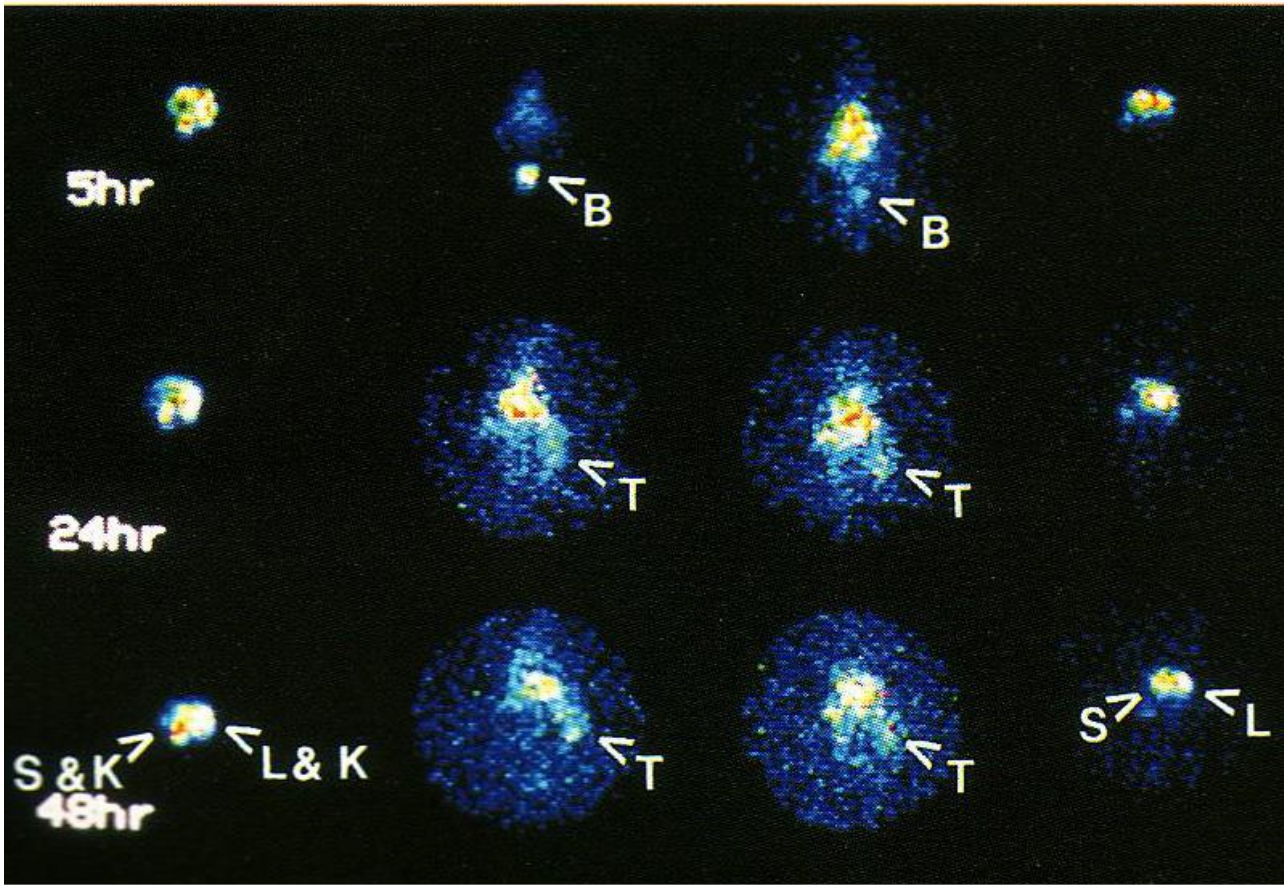




In^{111} isotope labelled polypeptides i.v. injection

The effect of the charge of amino acid X in XAK branched polypeptide on tissue distribution in mice with mammary carcinoma

carcinoma graft size:
1.8 x 1.2 cm



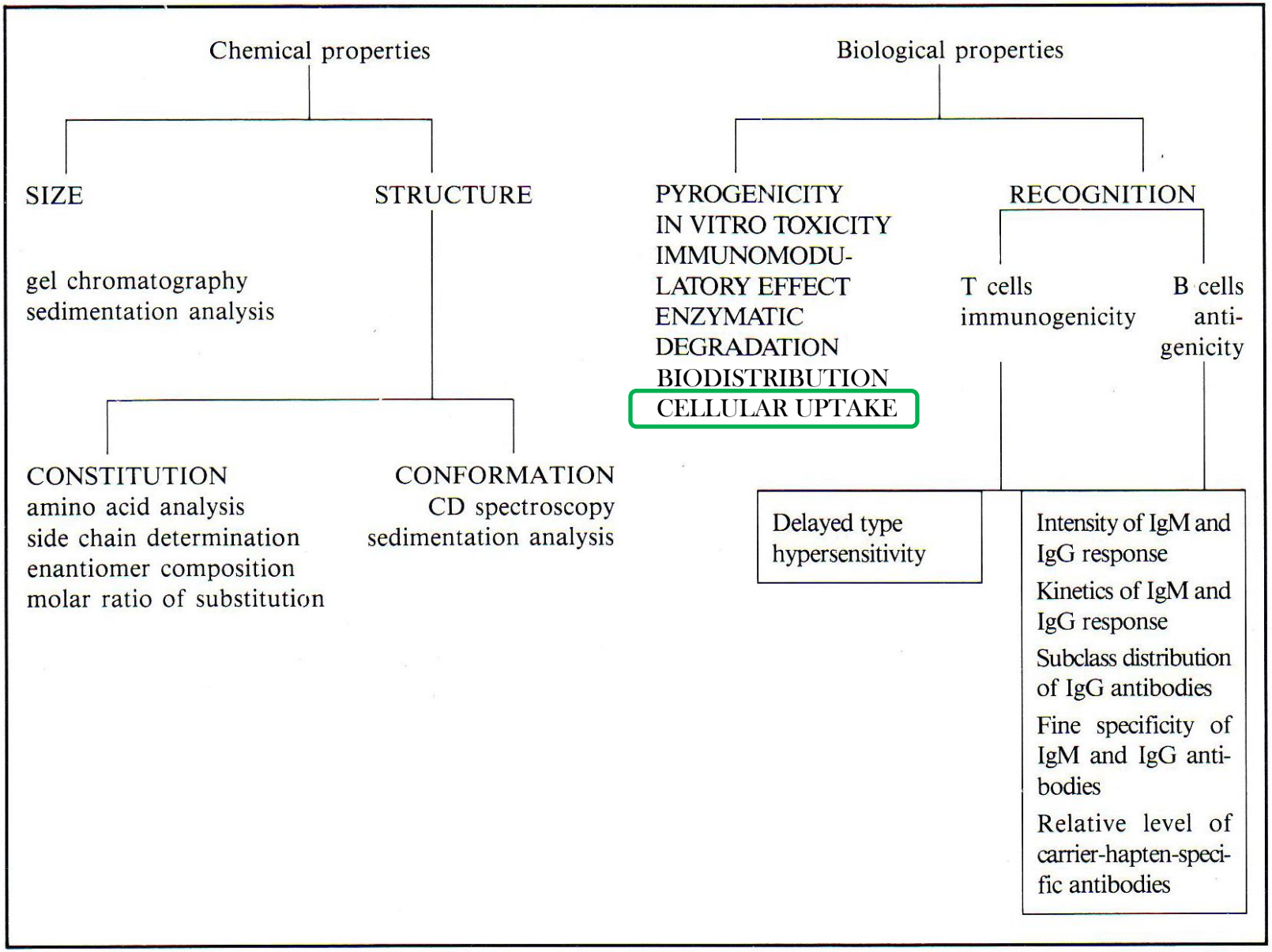
B = urinary bladder
T = tumour
S = spleen
K = kidney

AK Ac-EAK EAK Suc-EAK

In^{111} isotope labelled polypeptides i.v. injection

Annals of Nuclear Medicine Vol. 9, No. 4, 247–251, 1995

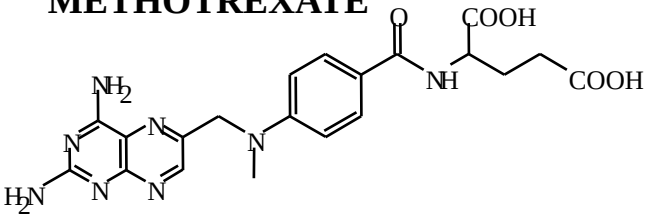
Characterization branched chain polypeptides



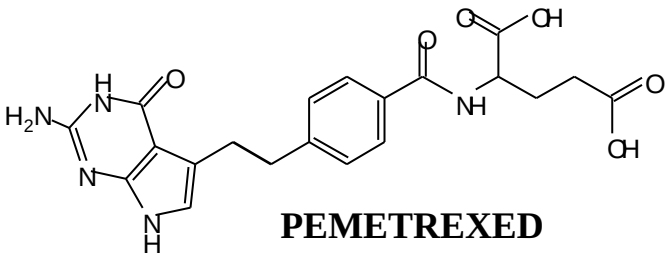
The effect of the identity and position of amino acid X on cellular uptake

Drug, epitope, reporter molecule

METHOTREXATE

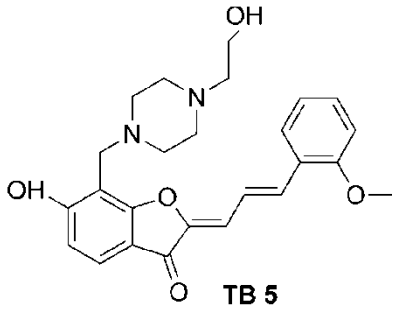


Hudecz F. et al. *Bioconjugate Chem.* **4:** 25 (1993)
Kóczán Gy. et al. *Bioconjugate Chem.* **13:** (2002)



PEMETREXED

Miklán Zs. et al.
J. Peptide Sci. 17: 805 (2011)



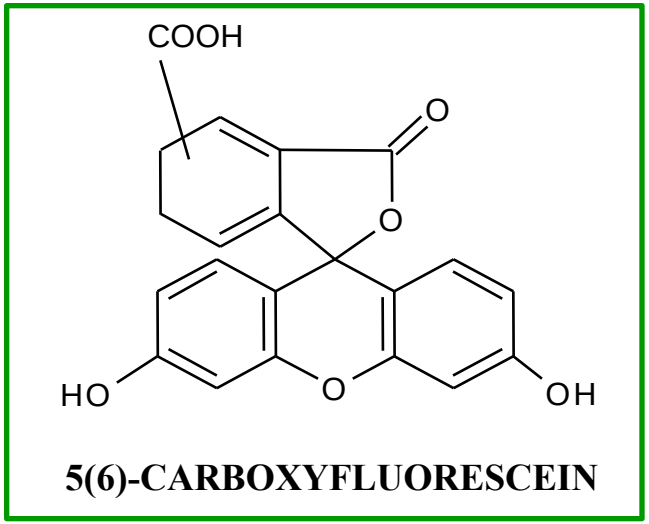
TB 5

Horvati, K.. et al.
Bioconjugate Chem. 22:981 (2012)

D-Trp-*D*-Cpa-*D*-Trp-Ser-Tyr-*D*-Lys-Leu-Arg-Pro-*D*-Ala

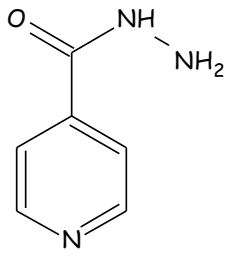
GN-RH ANTAGONIST, MI-1544

Mező, G. et al.*Bioconjugate Chem.* **7:** 642 (1996)
Vincze, B. et al. *J. Cancer Res. Clin. Onc.* **120:** 578 (1994)



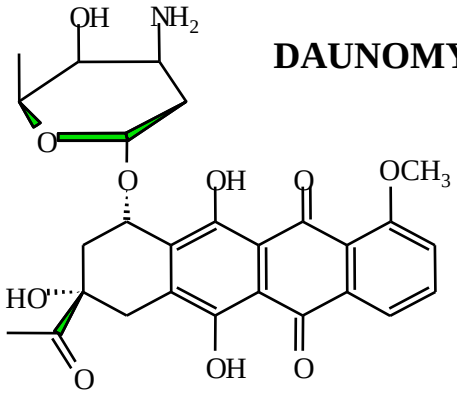
5(6)-CARBOXYFLUORESCEIN

Szabó R. et al. *Bioconjugate Chem.* 19: 1078 (2008)
Bánóczi Z. et al. *Bioconjugate Chem.* 19: 1375 (2008)



ISONIAZID

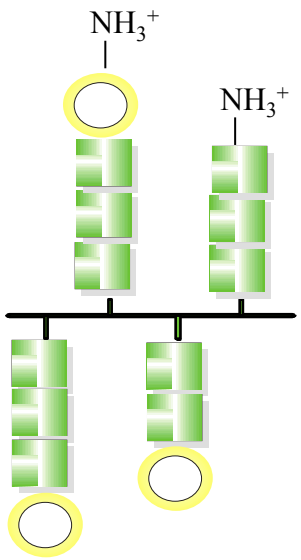
Horvati, K.. et al.
J. Peptide Sci. 15:385 (2009)



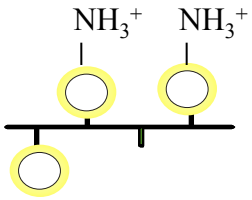
DAUNOMYCIN

Bánóczi Z. et al. *Archivoc* 143 (2008)
Miklán Zs. et al. Biopolymers 92:489 (2009)
Szabo R. et al. *BBA* 1798: 2209 (2010)

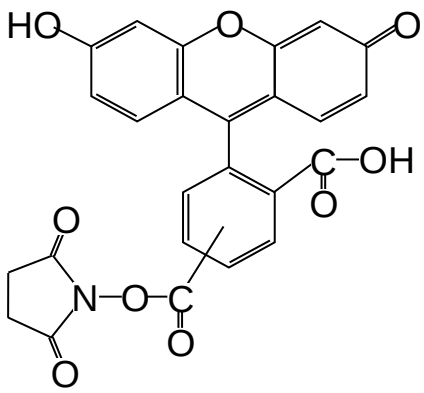
The effect of the identity and position of amino acid X on cellular uptake

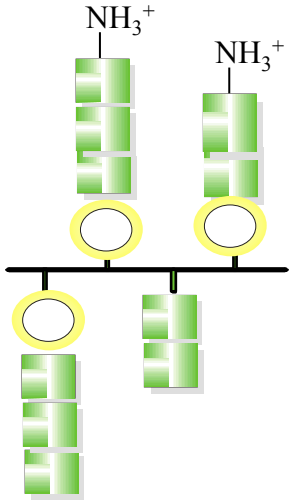


XAK, poly[Lys(X_i-DL-Ala_m)]



X_iK, poly[Lys(X_i)]

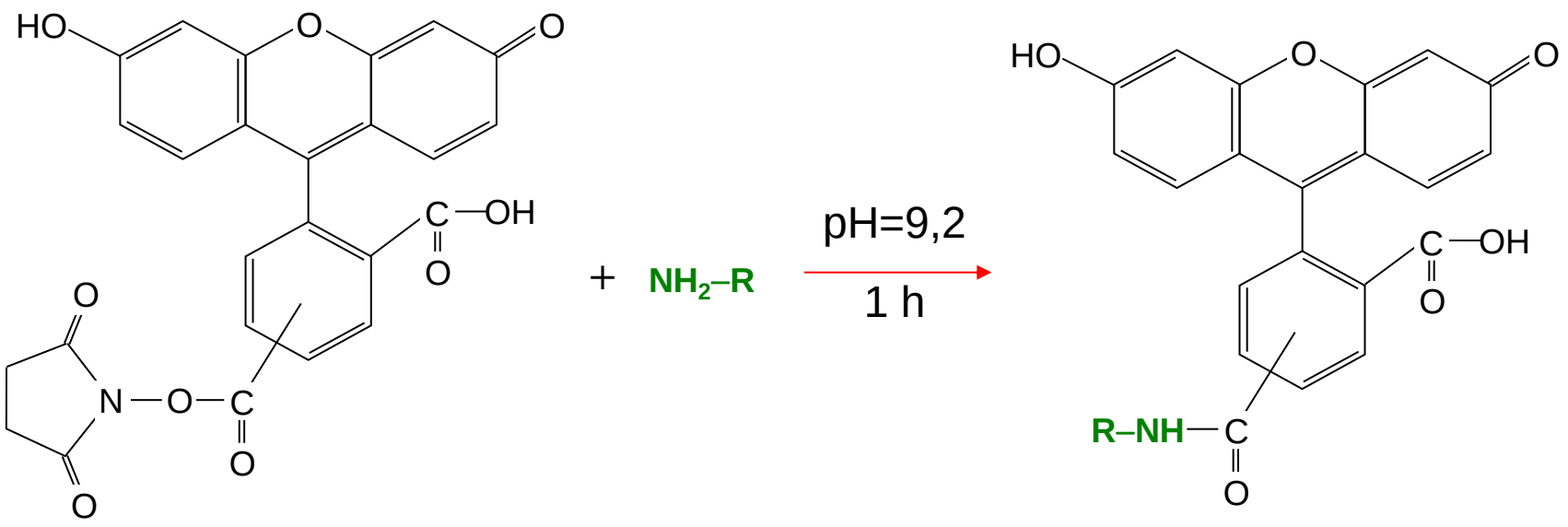




AXK, poly[Lys(DL-Ala_m-X_i)]

Hudecz F. et al. *J. Mol. Recognition* **16:** 288 (2003)
Szabó R. et al. *Bioconjugate Chemistry* **16:** 1442 (2005)
Szabó R. et al. *Bioconjugate Chem.* **19:** 1078 (2008)
Szabó, R., Sebestyén, M. et al. *ACS Combinatorial Science* 19: 246-254(2017)

Labelling branched polypeptides with 5(6)-carboxyfluorescein (CF)

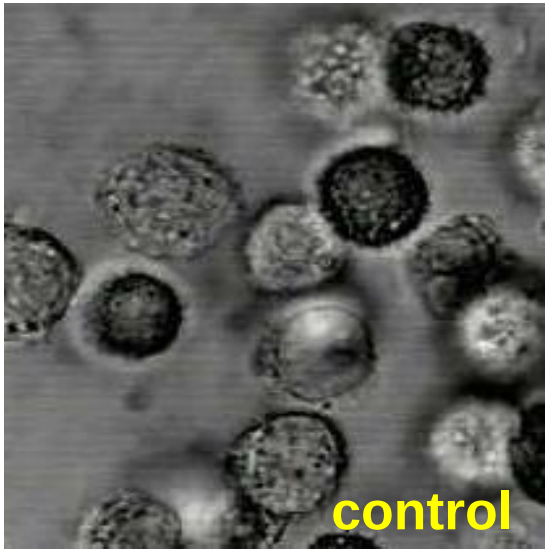


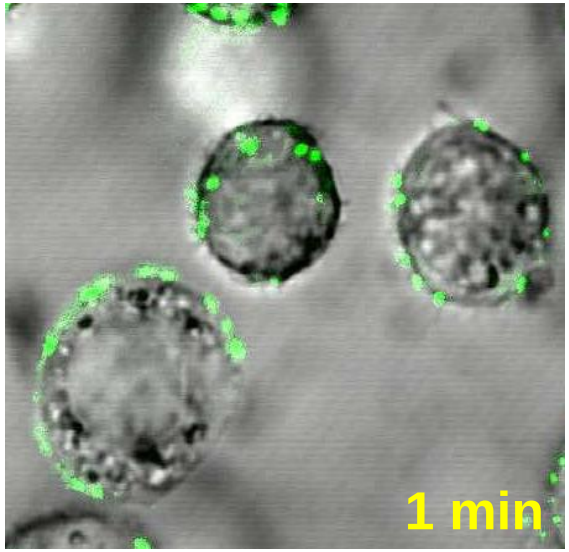
5(6)-carboxyfluorescein-succinimid-ester

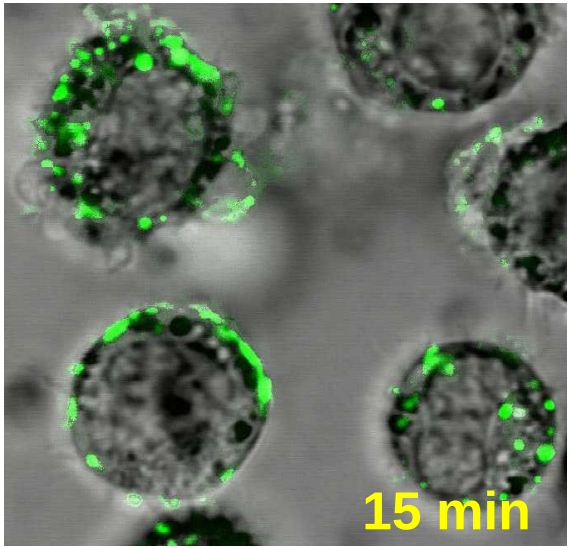
CF-polypeptide

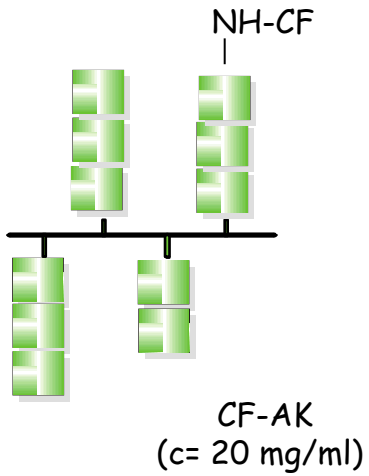
Time dependent cellular uptake of polypeptide AK

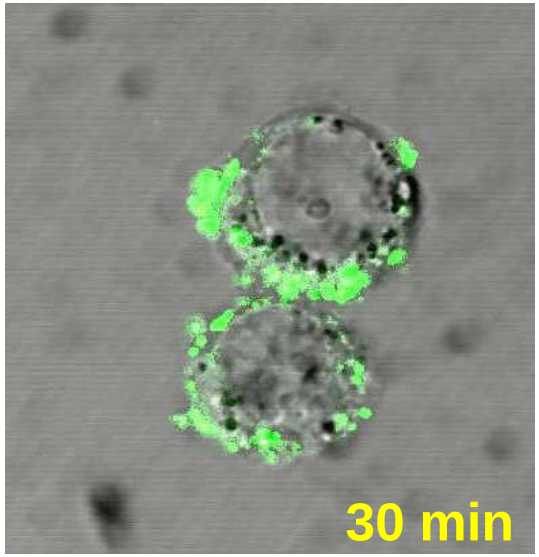
(J774 macrophage cells)

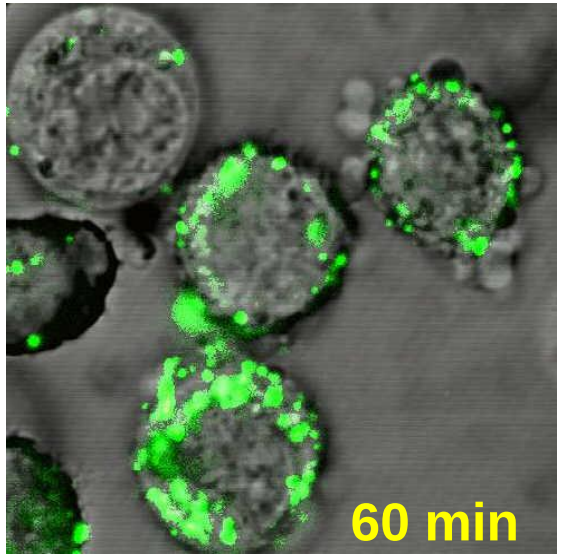






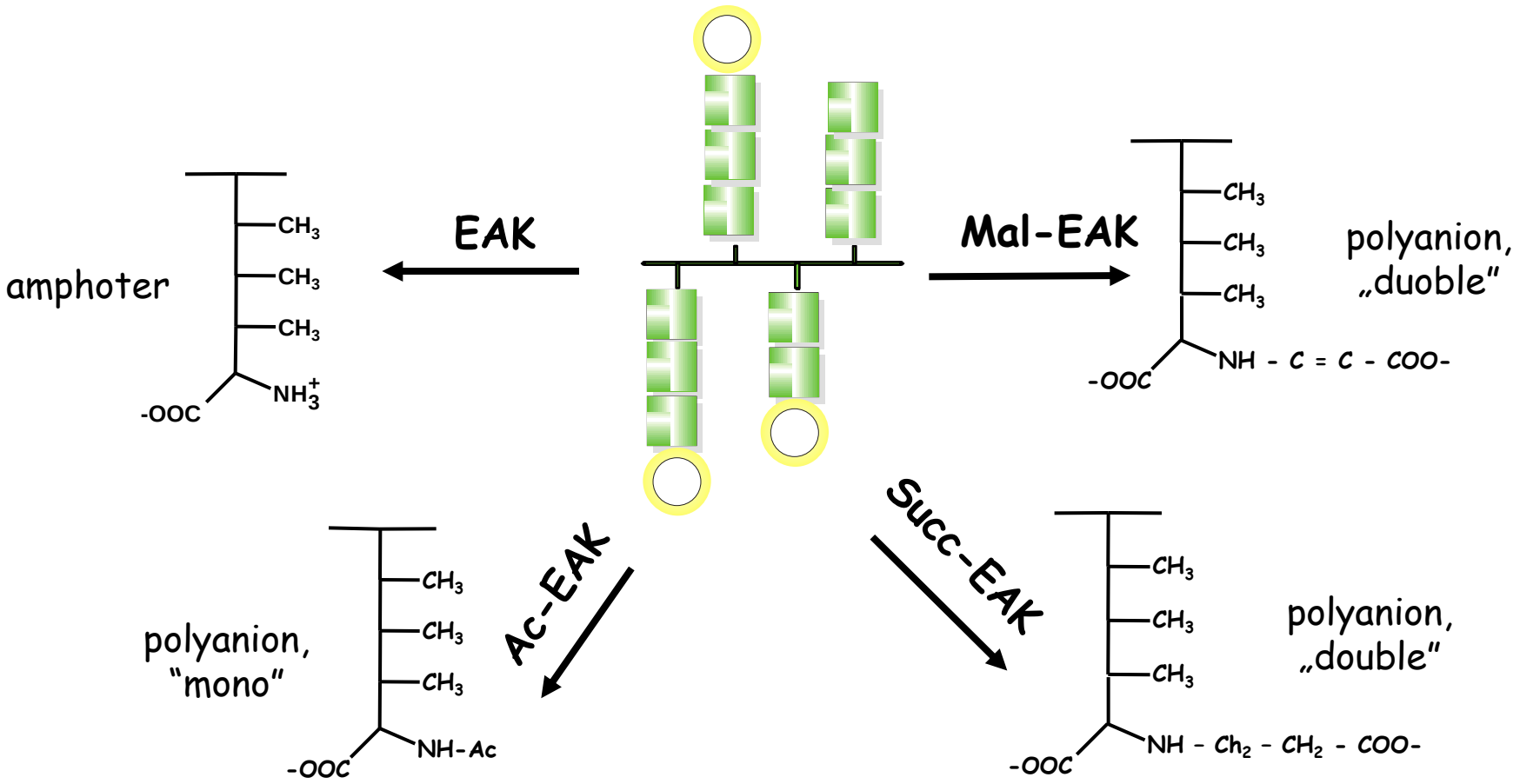




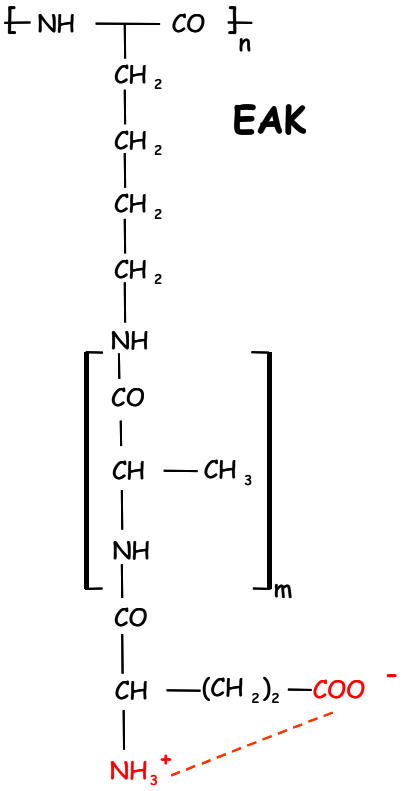


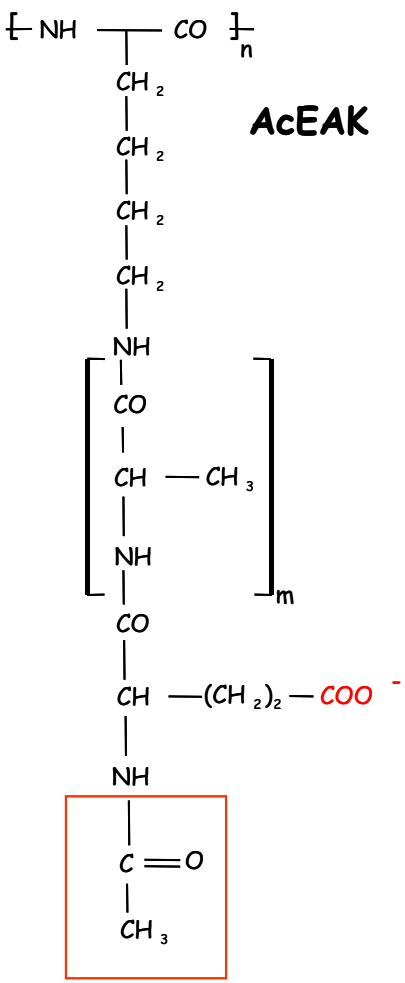
Szabó R. et al. *Bioconjugate Chem.* **19:** 1078 (2008)

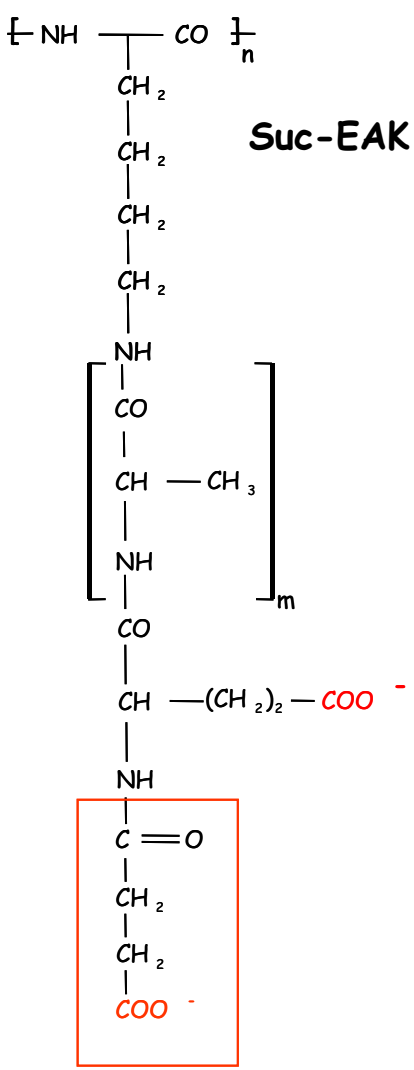
The effect of the identity and position of amino acid X on cellular uptake: amphotheric and polyanionic polypeptides

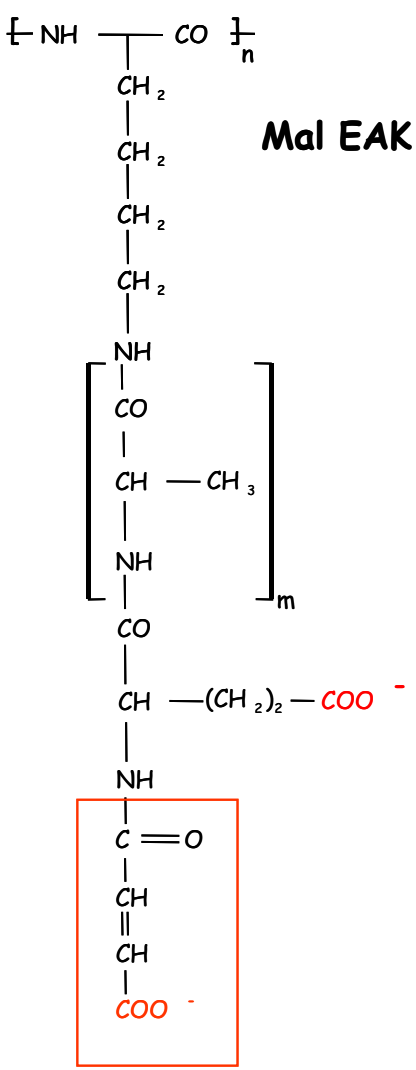


The structure of polypeptides

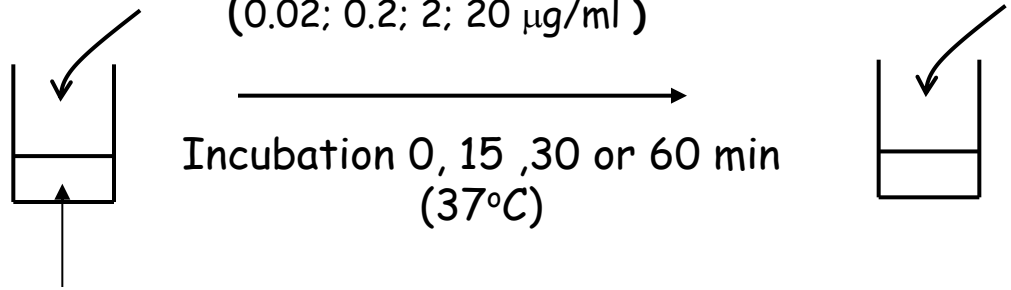








Protocol of the uptake studies

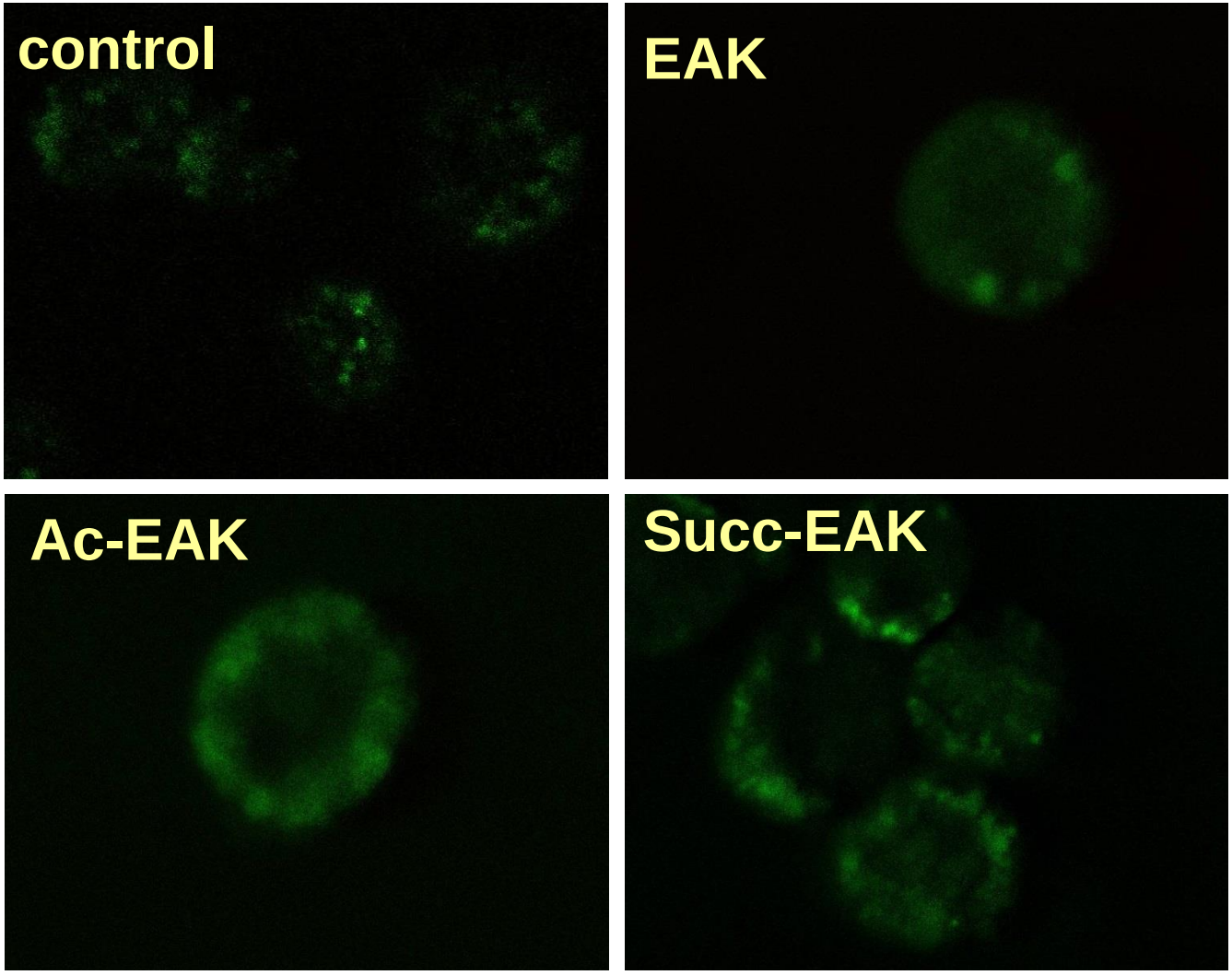


1. **0.5 % tryphan blue, 5 min**
2. **washing, PBS**

Fluorescence intensity (FACS)
(Beckton Dickinson , FACSCalibur, FL1)

Confocal microscopy
(BIO RAD MRC 1024, kripton-argon laser (λ_{ex}= 419 nm ,λ_{em}= 519 nm)

Uptake of amphoteric (EAK) and polyanionic branched polypeptides by J774 cells

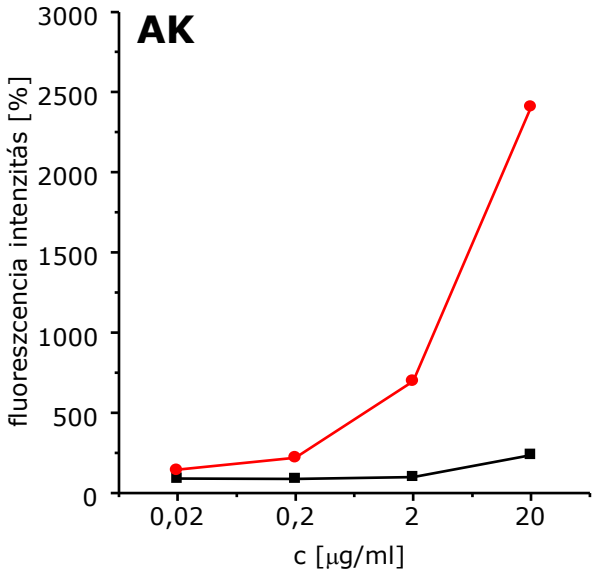


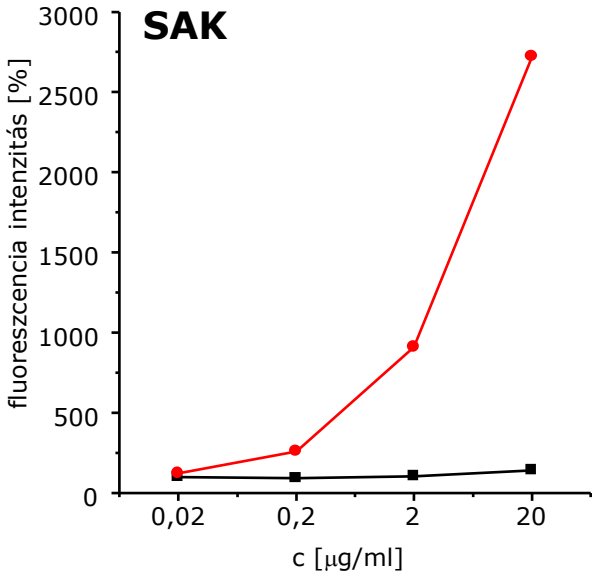
(20 μg/ml, 60 min)

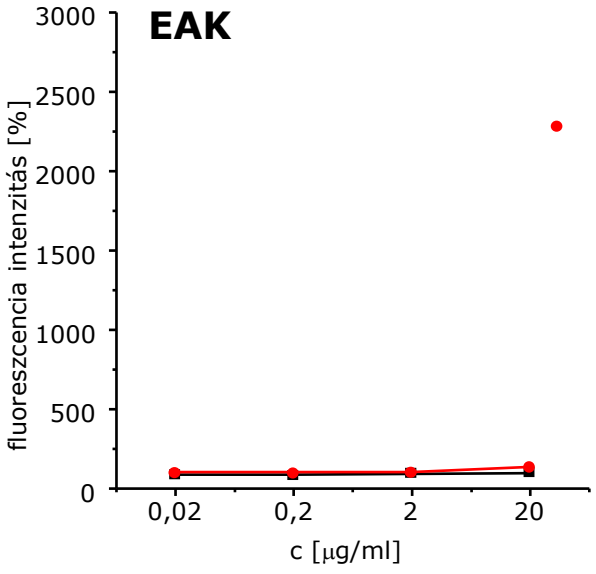
Uptake kinetics of polymers by fixed and living J774 cells

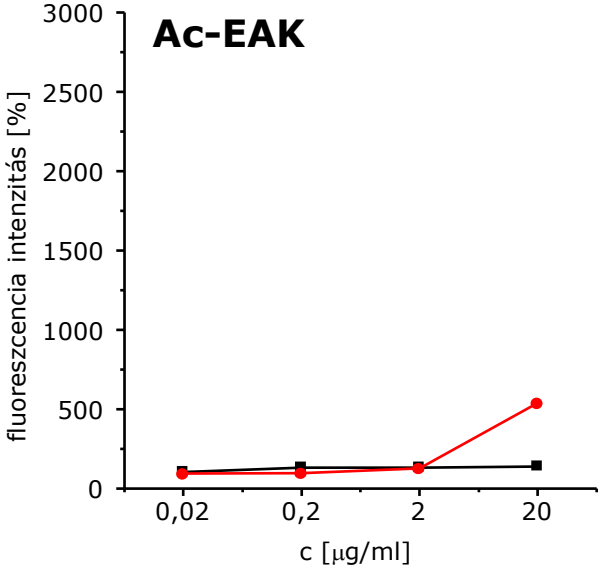
living cells

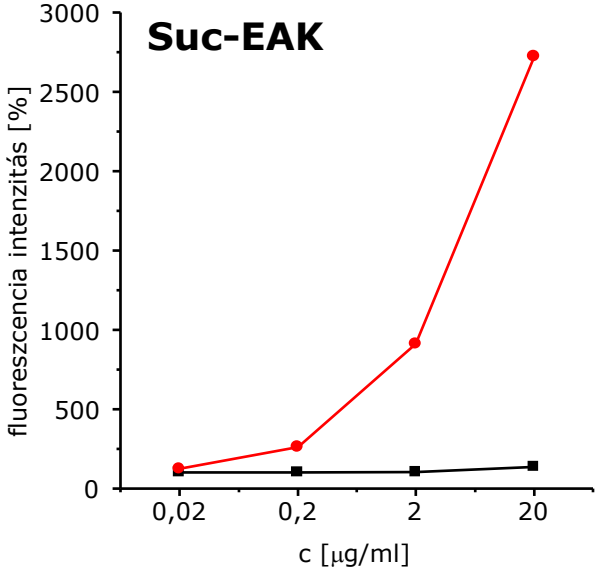
fixed cells by 4 % formalin



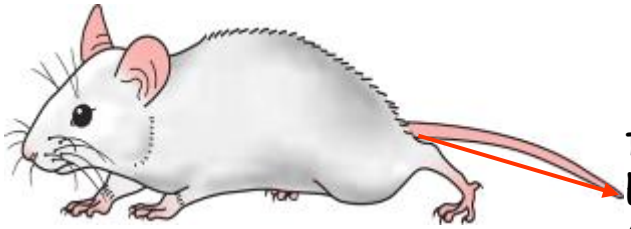




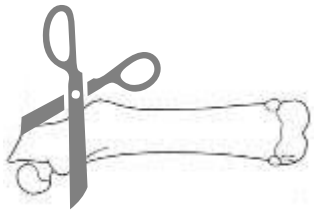




Isolation and differentiatiation of BMDM scavenger receptor [SR-A] (+/-)

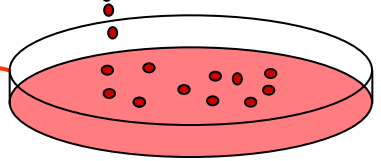


femur + tibia
EtOH 30 sec
(sterilisation)

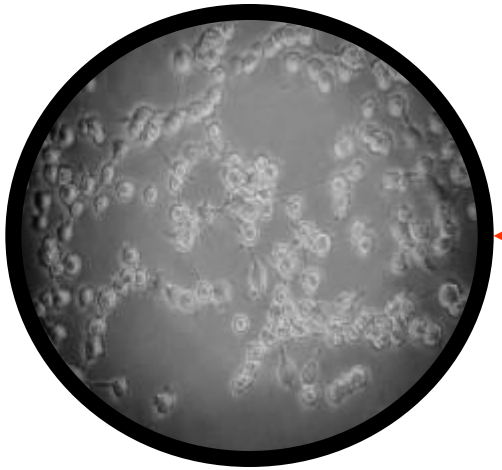


cutting off the joints

washing with PBS



2 weeks 37°C
Washing and
Feeding in
every 3rd day

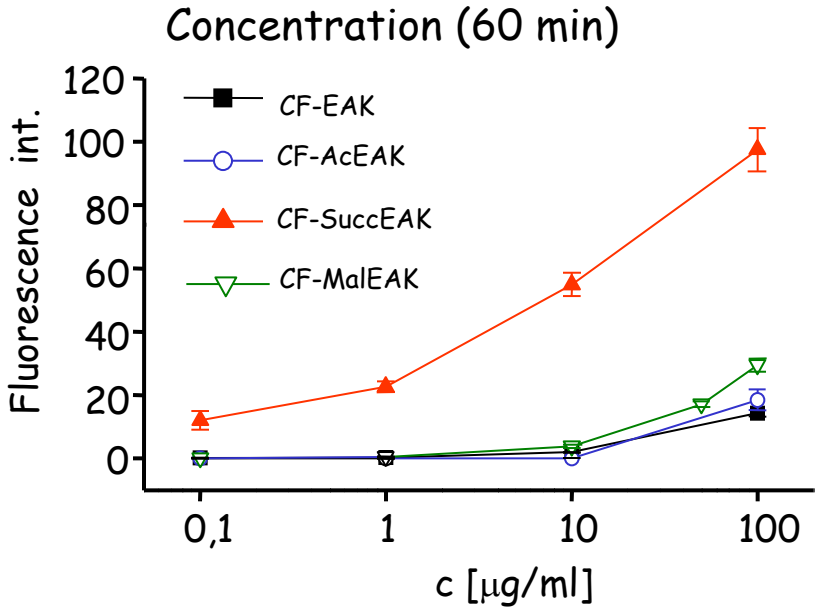


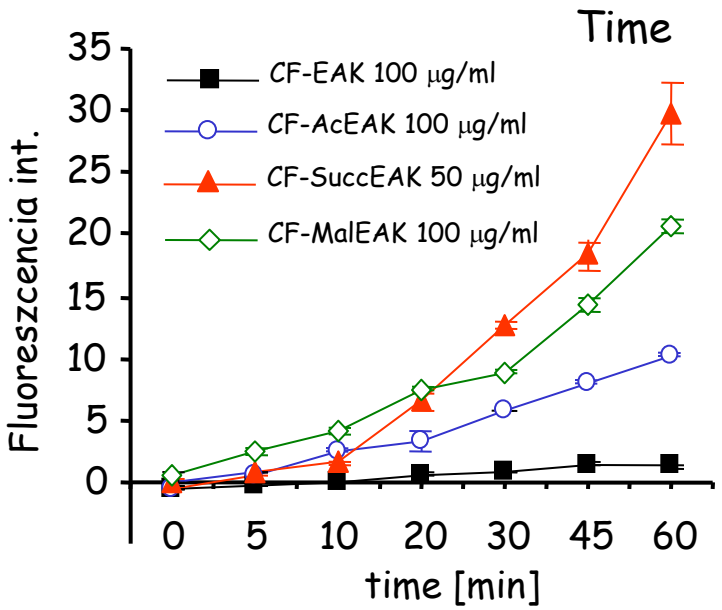
BP Petri dish:
adhesion via CR3

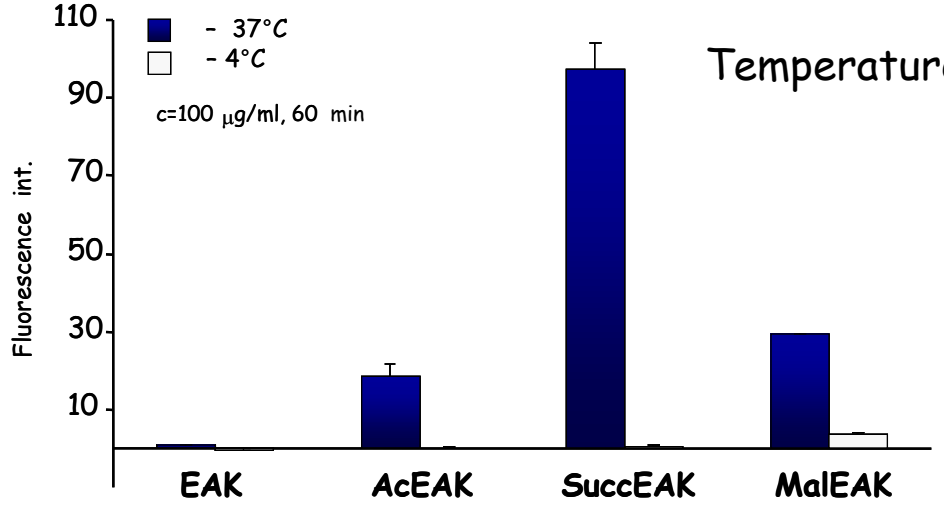
RPMI 1640
10% FCS (v/v)
0.16 mg/ml gentamycin
2 mM L-Gln
10 mM HEPES
10 ng/ml M-CSF

For uptake assays:
Plating into 24-well BP plate (10^5 cells/well)
24 hours prior to the experiment

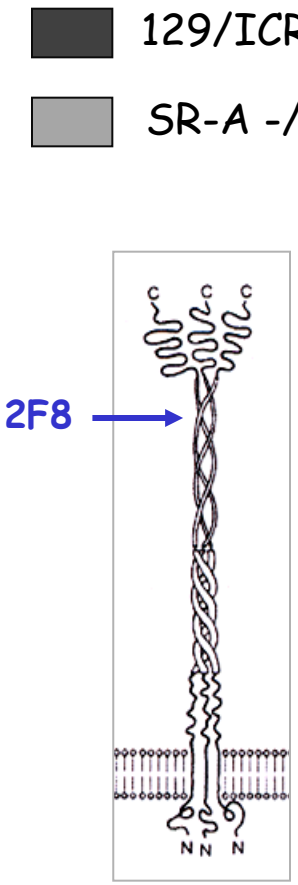
Uptake of CF-polyanionic polypeptides by BMDM macrophages

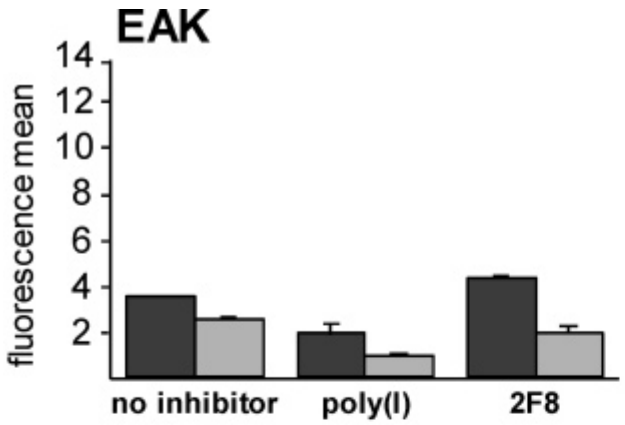


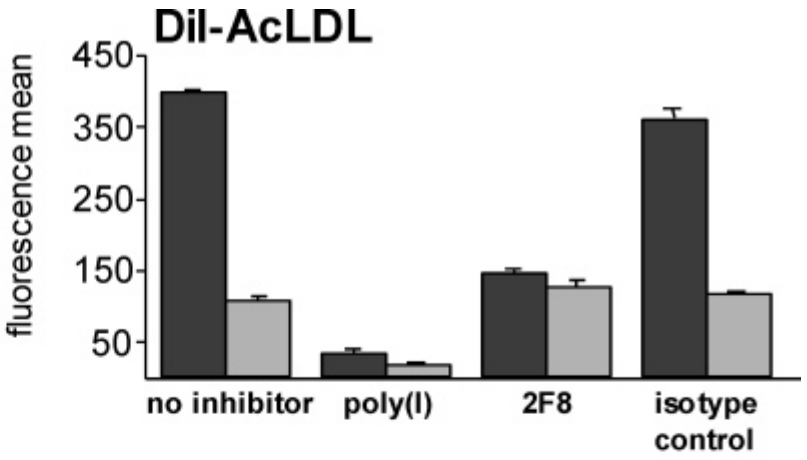


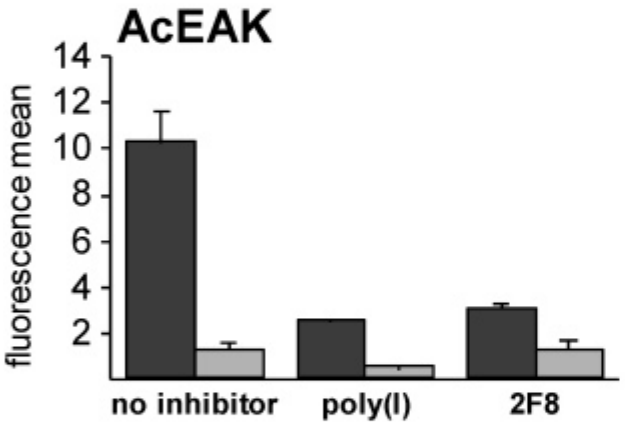


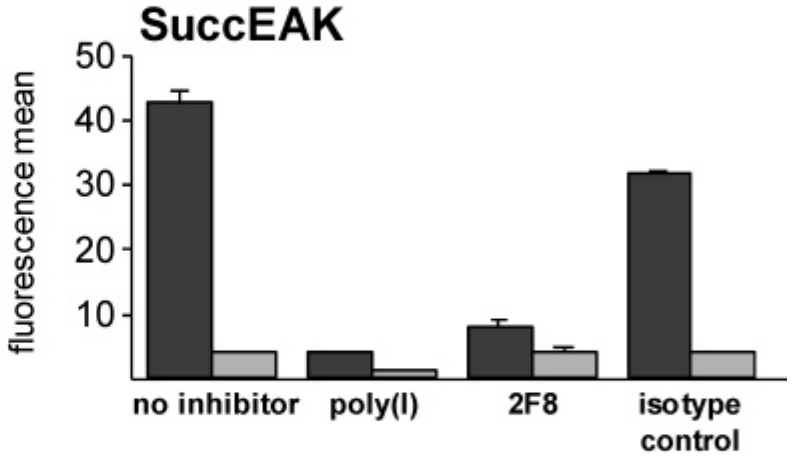
Inhibition of the SR-A mediated uptake of CF-polypeptides by receptor specific mAb or poly(I) in macrophages











Pre treatment with poly(I) (c = 50 μg/ml) or MAb 2F8 (c = 15 μg/ml) for 30 min
Cells were fixed with 4% formaldehyde

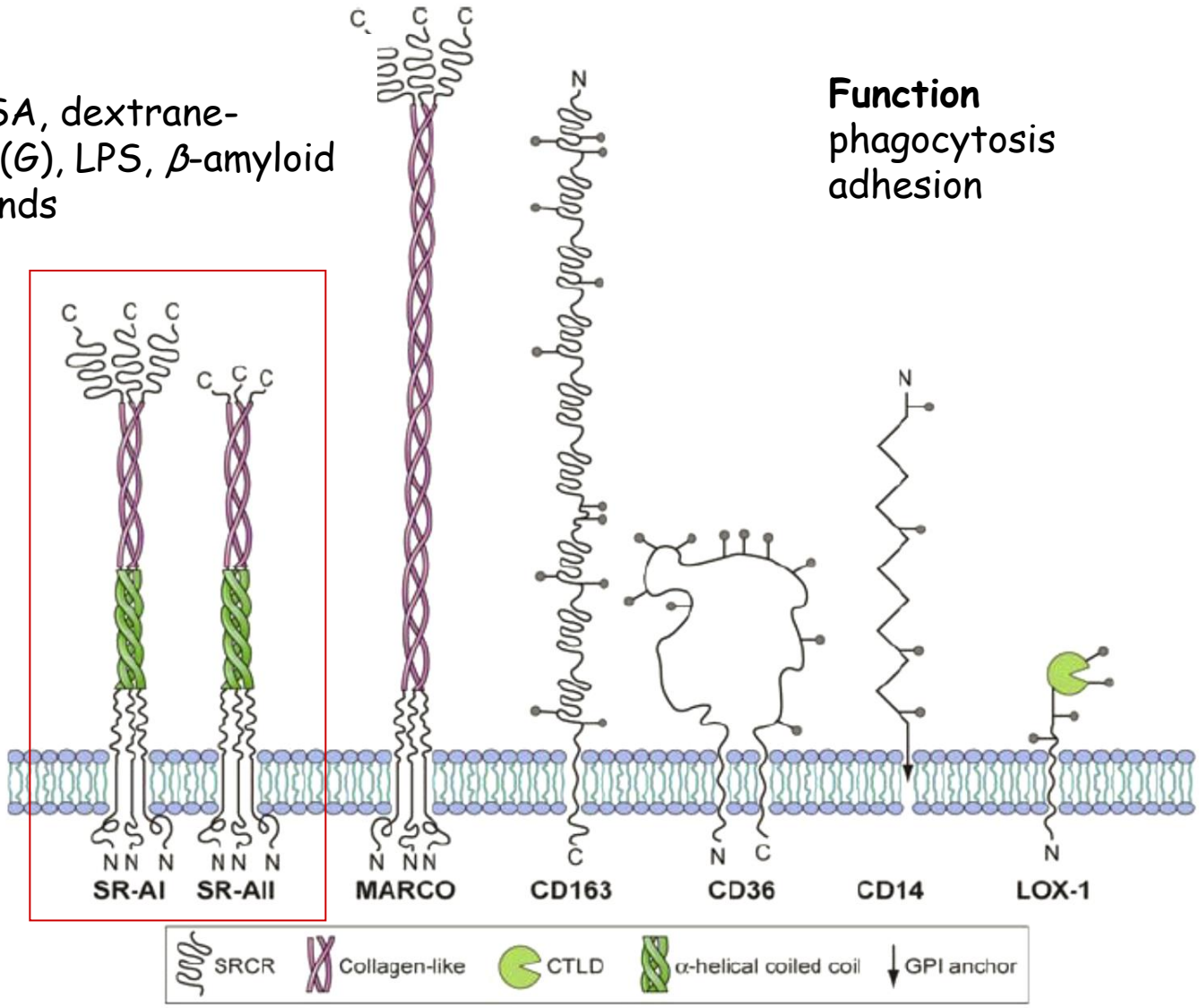
Targeting macrophages via scavenger receptor

Ligands
OxLDL, AcLDL, malBSA, dextrane-sulphate, poly(I), poly(G), LPS, β-amyloid
⇒ polyanionic compounds

Function
phagocytosis
adhesion

Expression
on macrophages, dendritic cells and endothelial cells

cell specificity

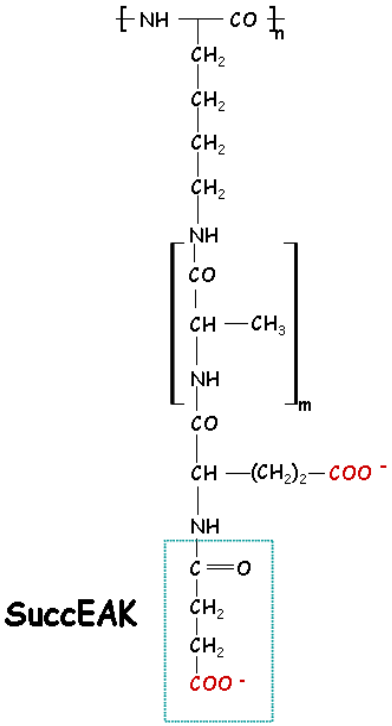


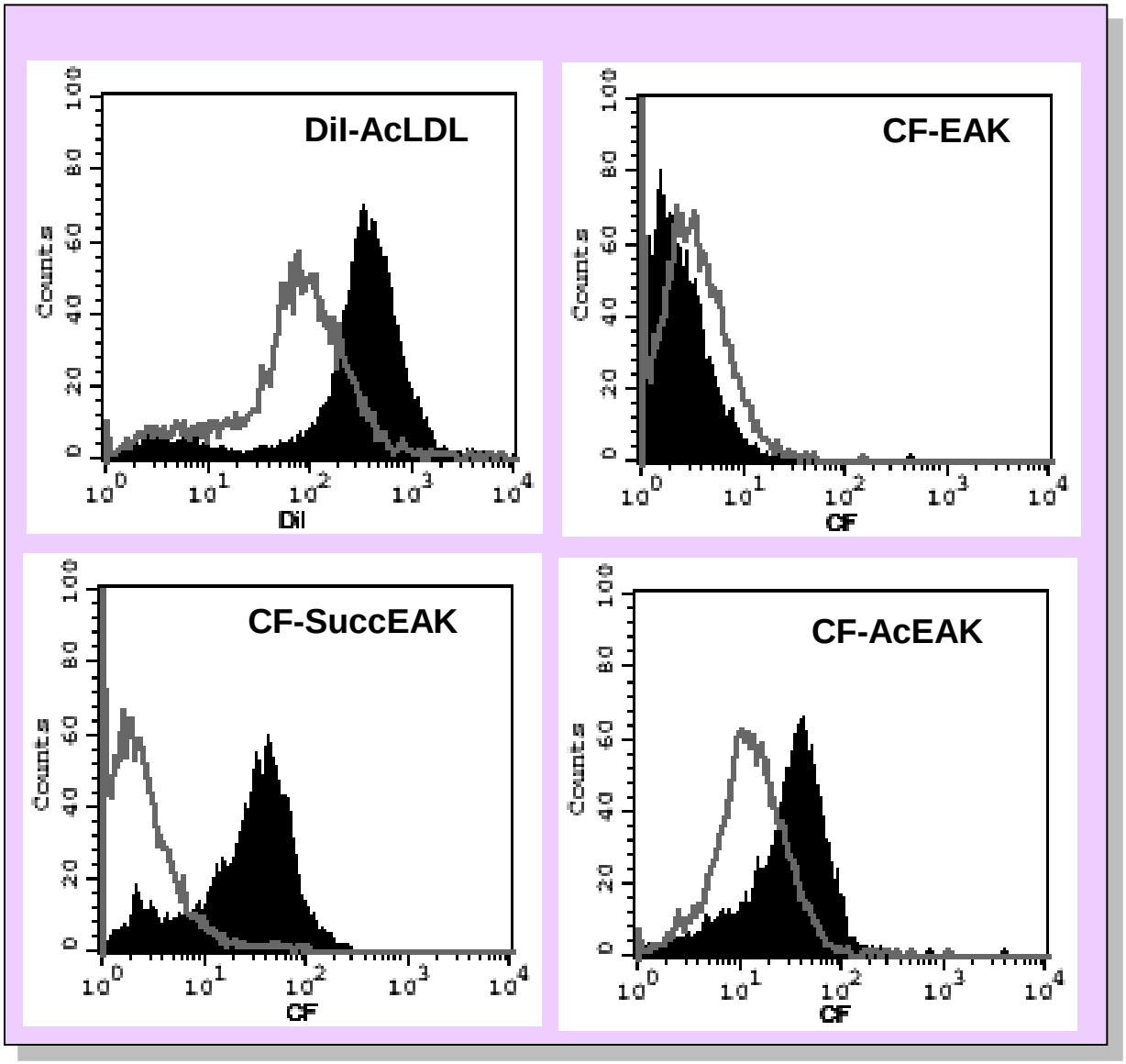
Taylor, P. R. et al, *Annu. Rev. Immunol.* (2005) 23: 901-44
Becker, M.et al, *Eur J Immunol.* (2006) 36: 950-60

Uptake of polyanionic polypeptides by macrophages via SR-A

wt (129/ICR)

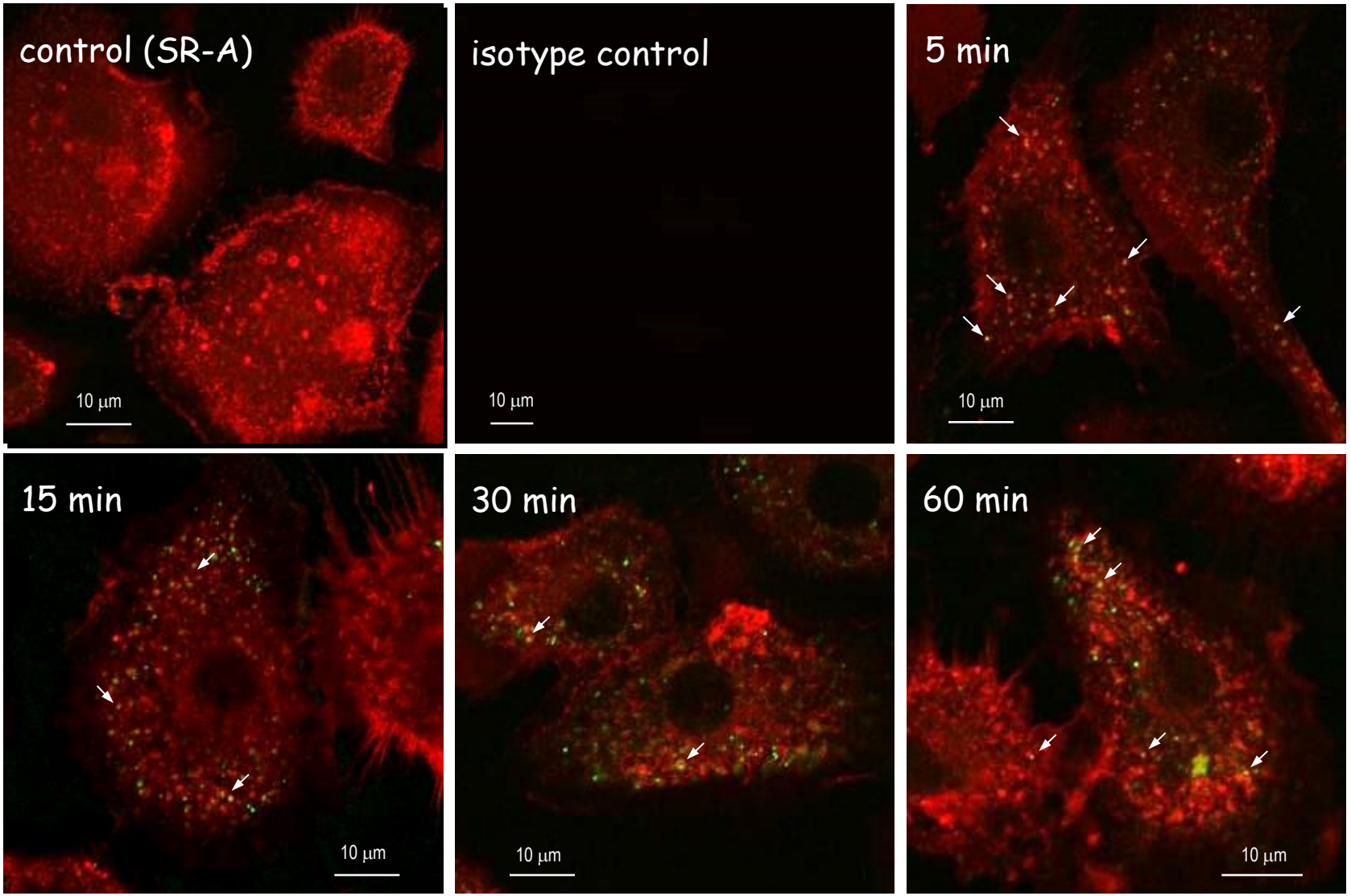
SR-A -/-





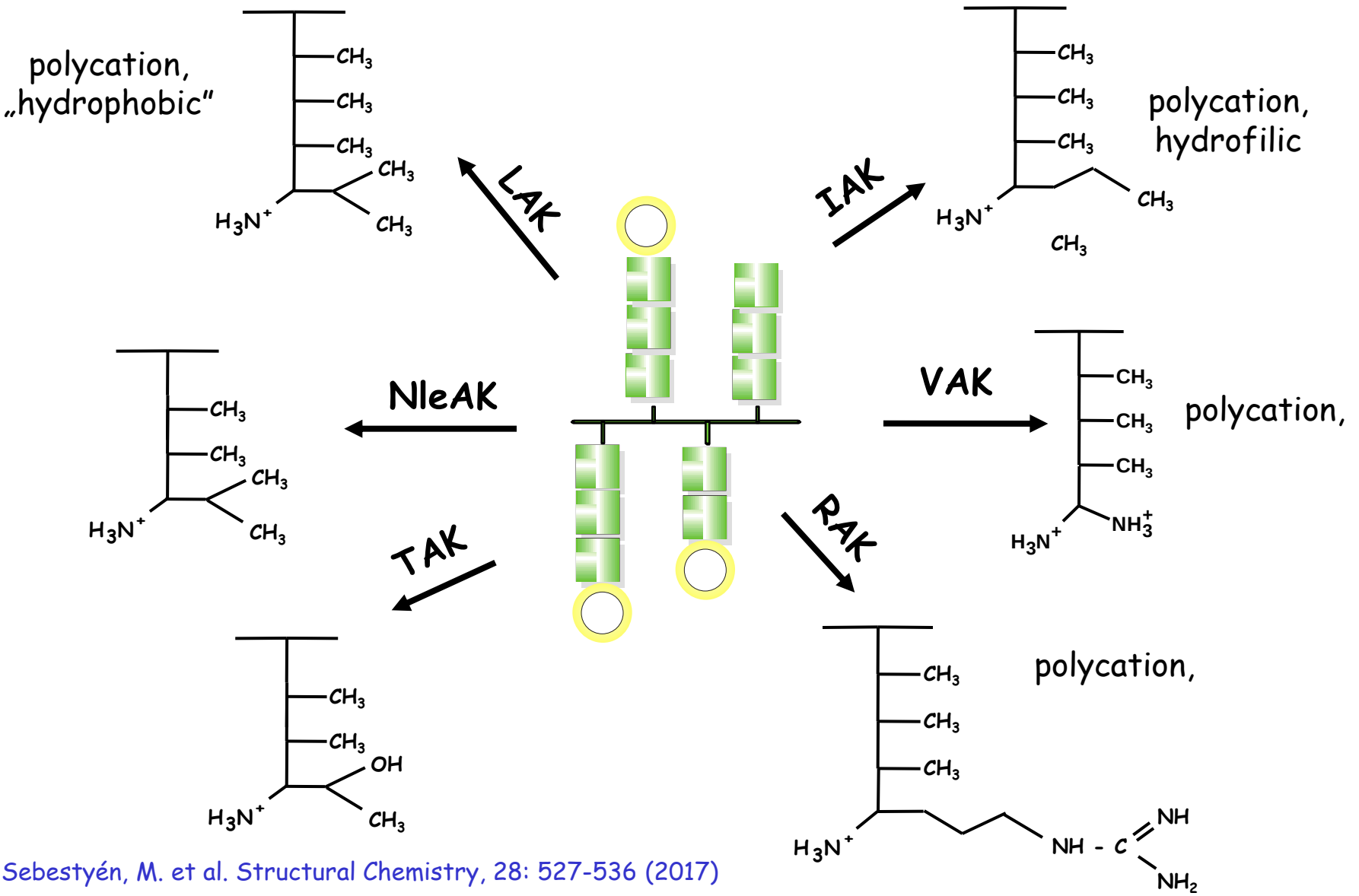
Szabó, R. et al, *Bioconj. Chem.* (2005) 16:1442-1450

Uptake of polyanionic polypeptides by bone marrow derived macrophages via SR-A



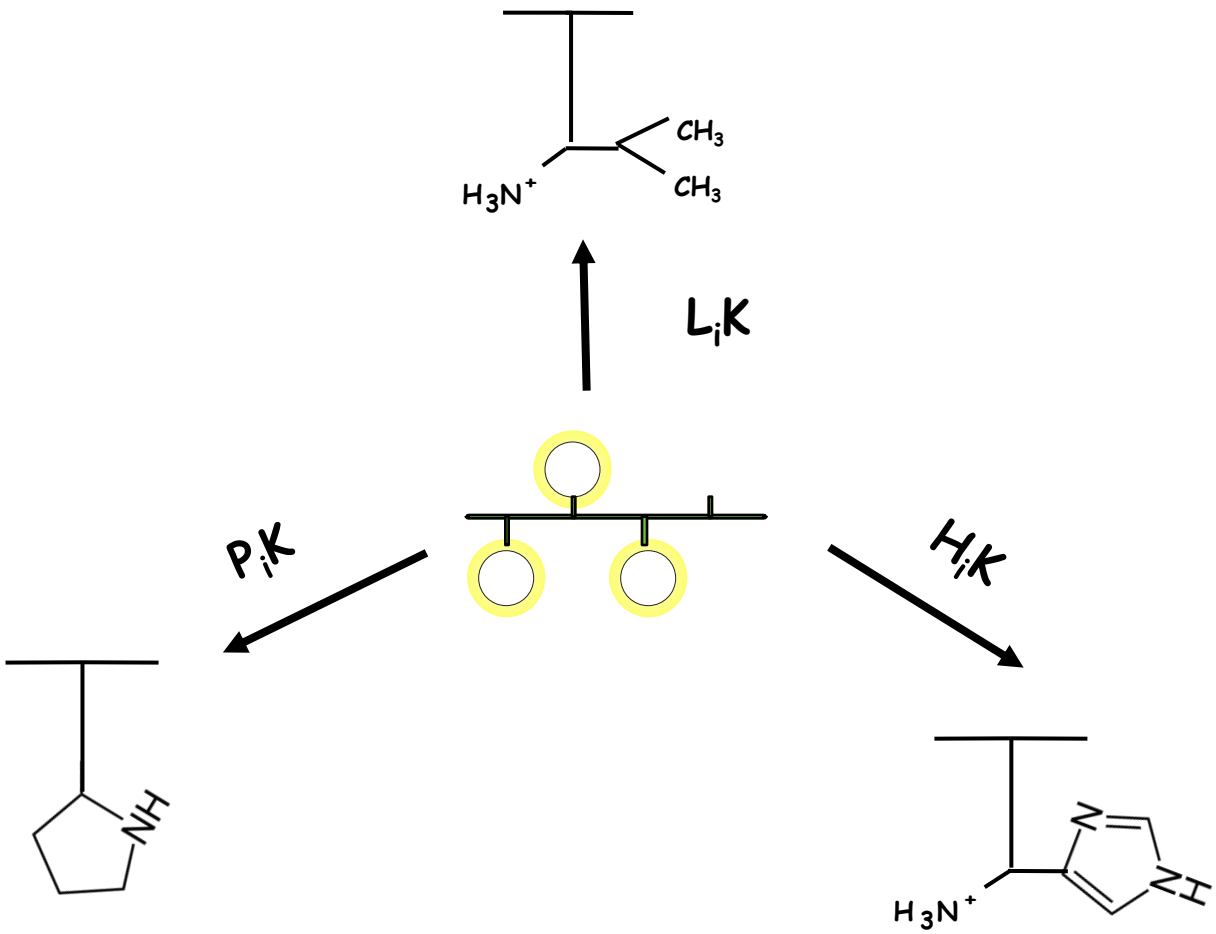
Colocalisation of SR-A (2F8) with CF-SuccEAK

The effect of the identity of amino acid X on cellular uptake of polycationic XAK polypeptides



Sebestyén, M. et al. Structural Chemistry, 28: 527-536 (2017)

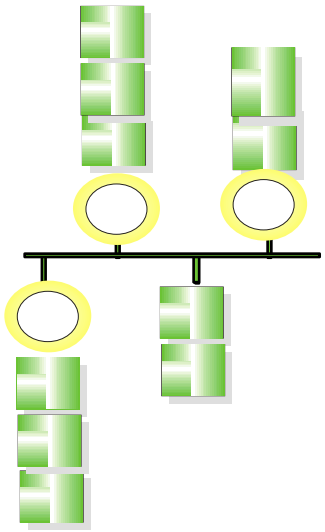
The effect of the identity of amino acid X on cellular uptake: polycationic X_iK polypeptides

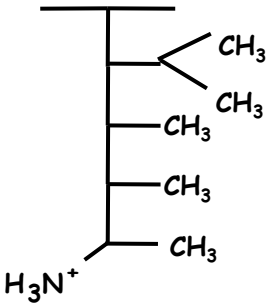


Sebestyén, M. et al. Structural Chemistry, 28: 527-536 (2017)

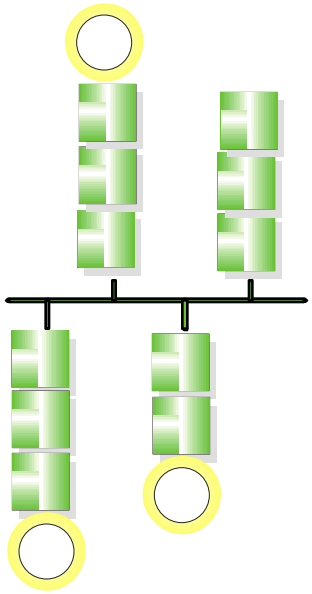
The effect of the position of amino acid X on cellular uptake: polycationic polypeptides

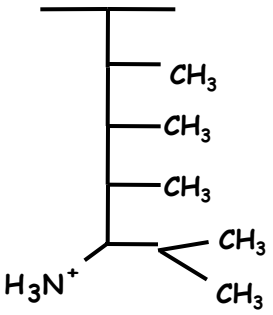
ALK



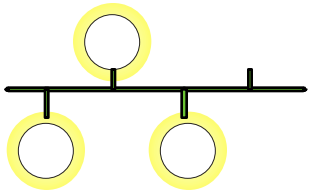


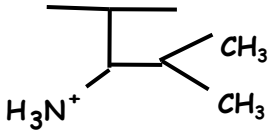
LAK





L_iK





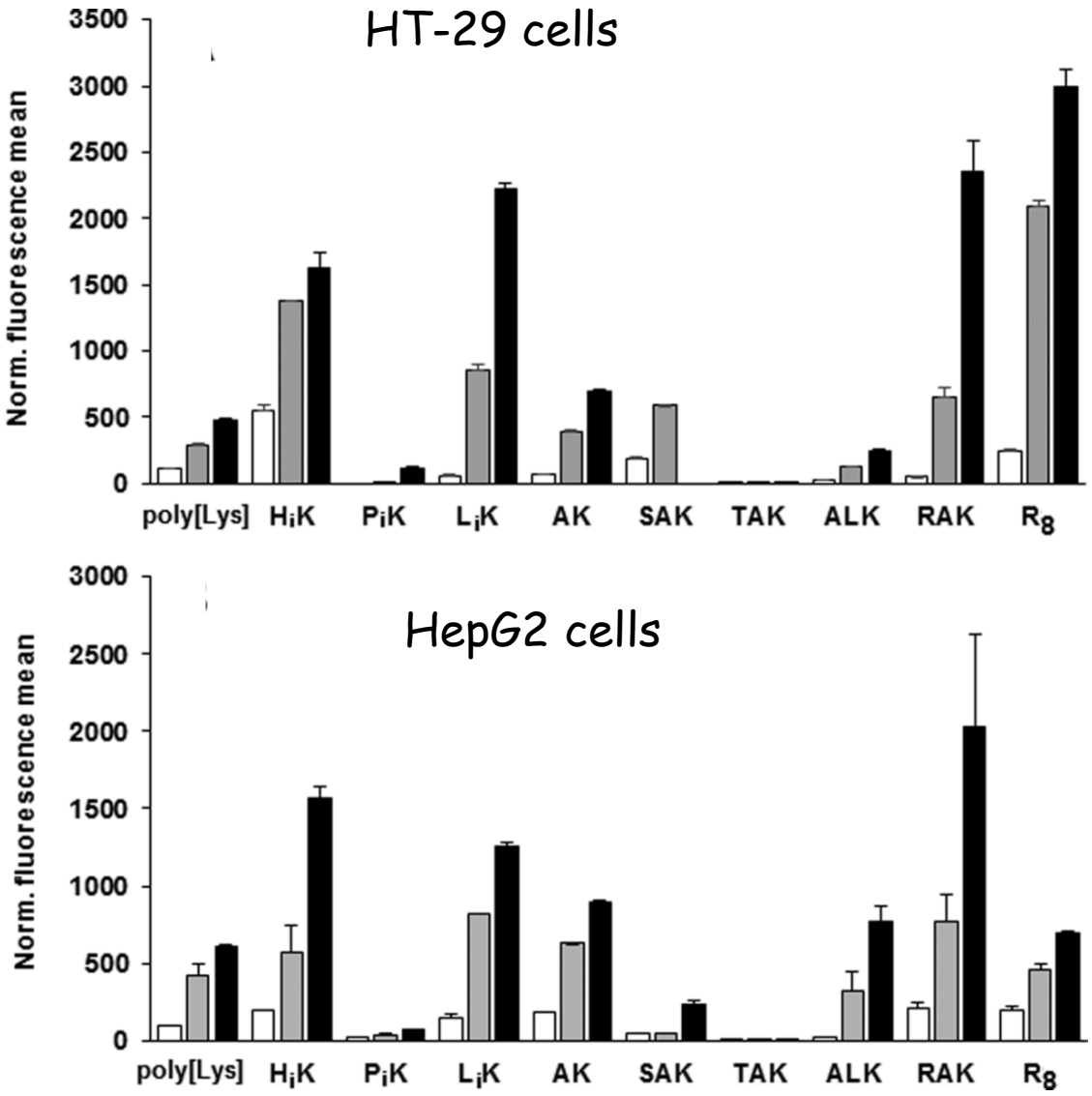
The effect of the identity of amino acid X and of cell type on cytotoxicity: polycationic polypeptides

LC_{50} [μg/ml]

polypeptide	HT-29	HepG2
poly[Lys]	44.1±4.6	32.3±10.8
AK	>100	>100
SAK	>100	>100
TAK	>100	>100
H_iK	99,9	>100
P_iK	>100	>100
L_iK	>100	>100

Szabó, R., Sebestyén, M. et al. *ACS Combinatorial Science* 19: 246-254(2017)

Concentration dependent uptake of the CF-polypeptides



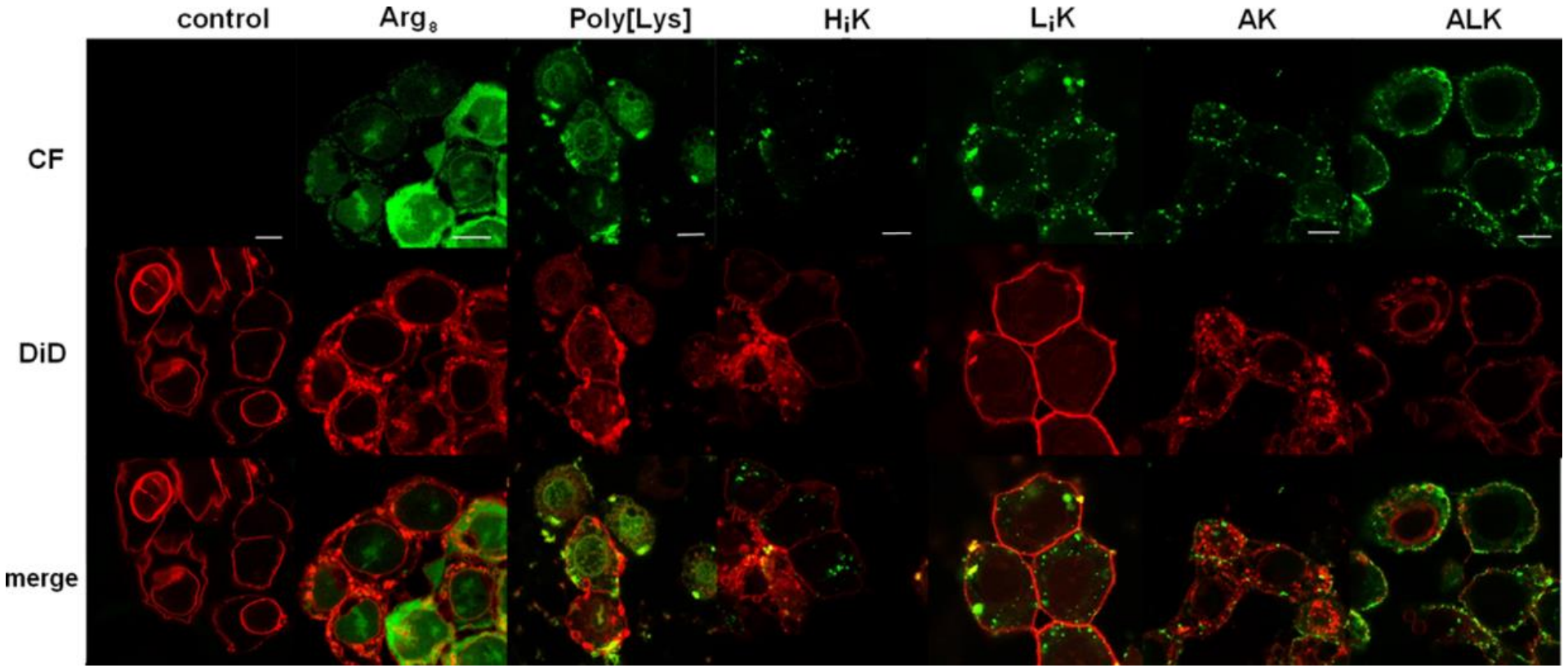
(White) c = 10 µg/mL.
(Gray) c = 50 µg/mL.
(Black) c = 100 µg/mL.

60 min

Data were normalized to carboxyfluorescein content of each Cf-(poly)peptide.

Intracellular localization of CF-polypeptides and Arg_8 in HT-29 human colon carcinoma cells

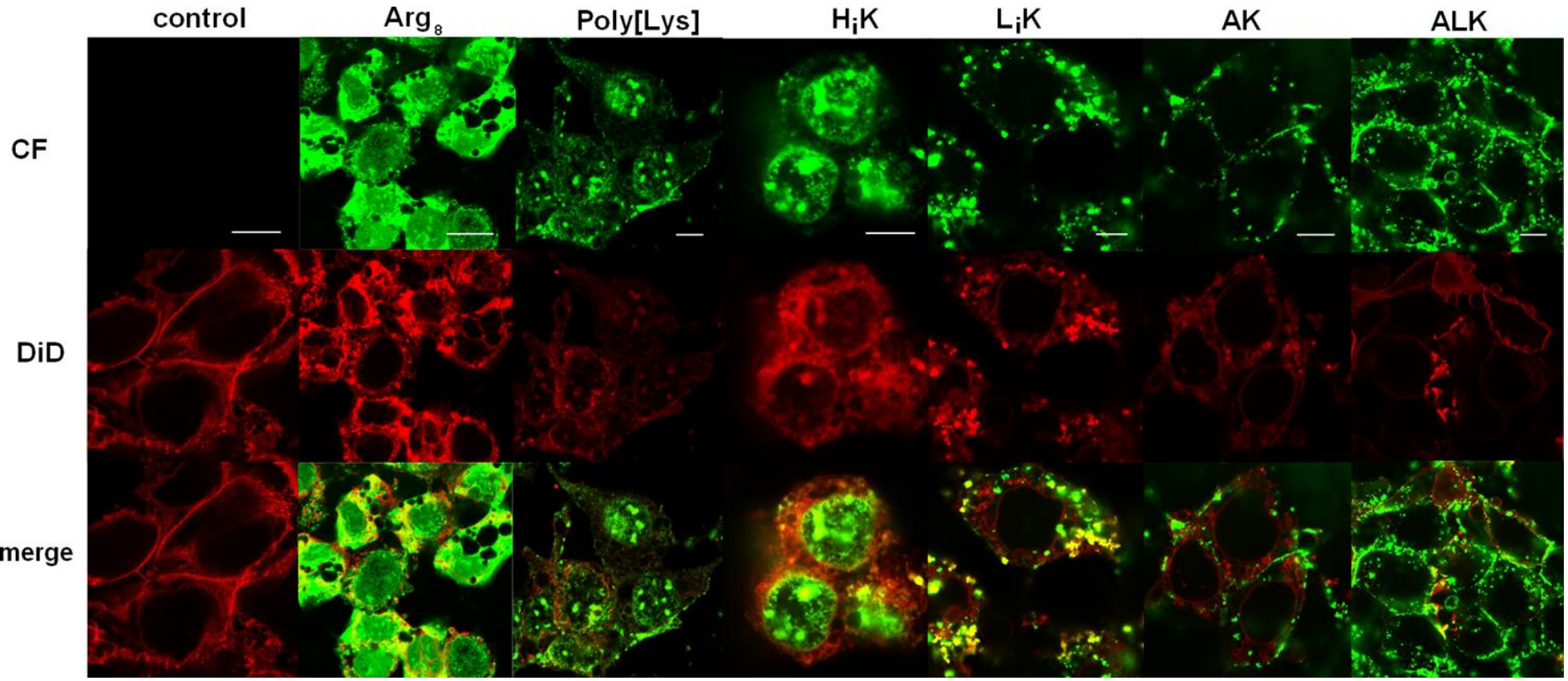
c = 100 μg/mL after 60 min



Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4).
Membrane structures were labeled with DiD perchlorate (red). Each bar represents 10 μm.

Szabó, R., Sebestyén, M. et al. *ACS Combinatorial Science* 19: 246-254(2017)

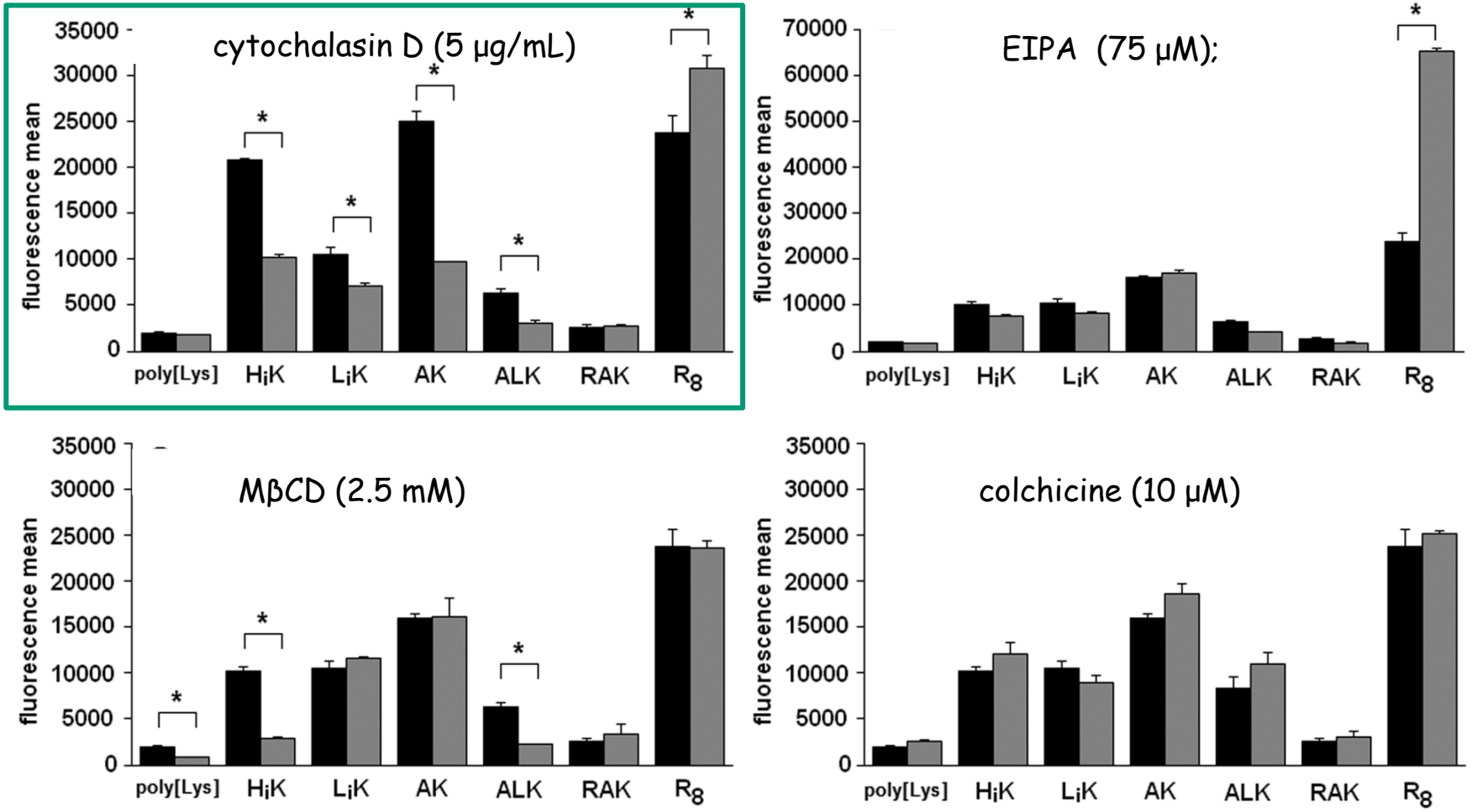
Intracellular localization of CF-polypeptides and Arg_8 in HepG2 human hepatocarcinoma cells



Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4).
Membrane structures were labeled with DiD perchlorate (red). Each bar represents 10 μm.

Szabó, R., Sebestyén, M. et al. *ACS Combinatorial Science* 19: 246-254(2017)

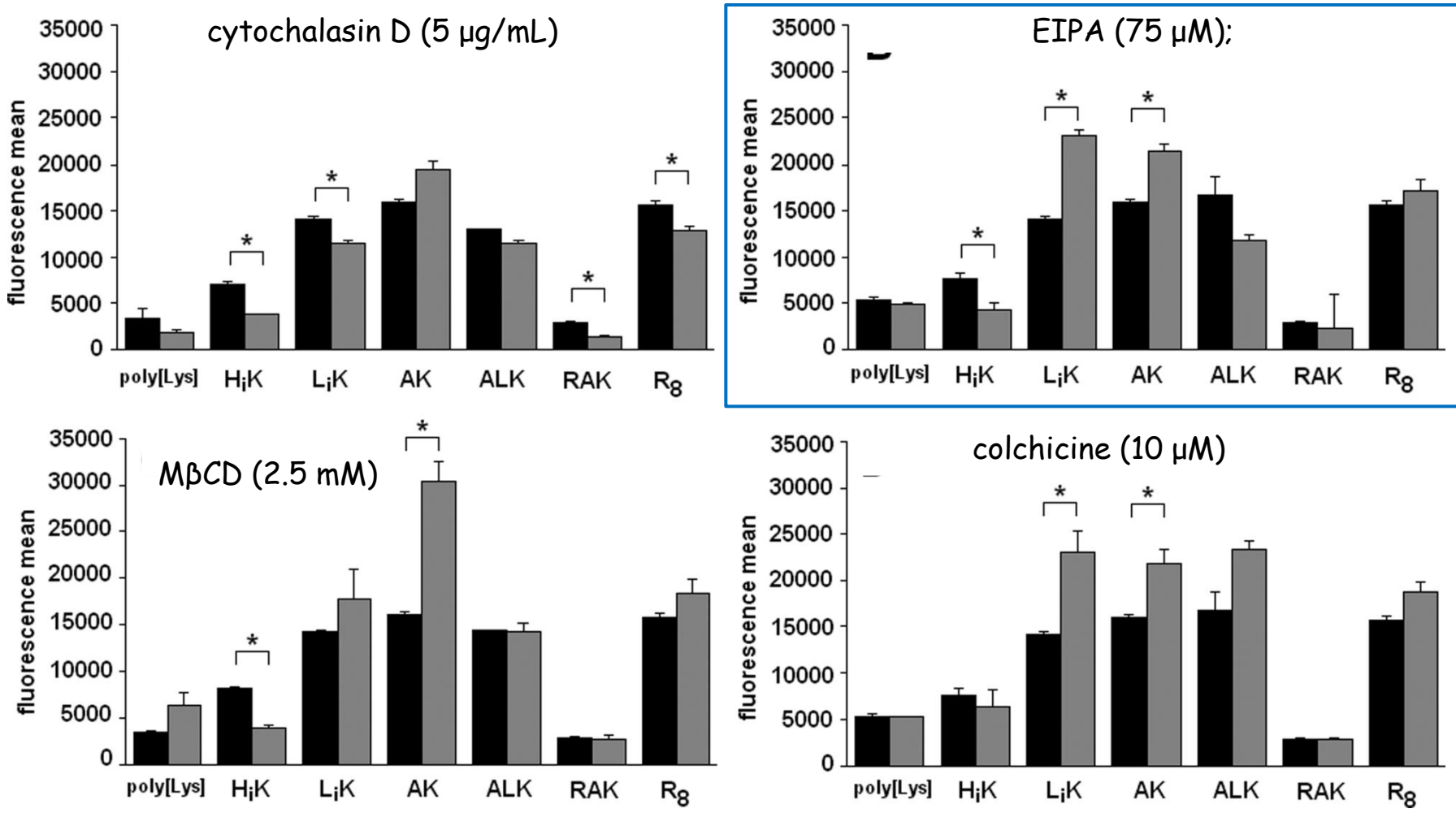
Uptake of the CF labeled polypeptides and Arg_8 by HT-29 cells in the presence of endocytosis inhibitors



Black: CF-polypeptides (c = 100 µg/mL; CF-poly[Lys], c = 50 µg/mL) and Cf-Arg_8 (R8, c = 50 µg/mL) without inhibitor. Average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control. Statistical analysis by Student's t test; *p < 0.05

Szabó, R., Sebestyén, M. et al. *ACS Combinatorial Science* 19: 246-254(2017)

Uptake of the CF labeled polypeptides and Arg_8 by HepG2 cells in the presence of endocytosis inhibitors



Black: CF-polypeptides (c = 100 μg/mL; CF-poly[Lys], c = 50 μg/mL) and CF-Arg_8 (R8, c = 50 μg/mL) without inhibitor.

Average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control.

Statistical analysis by Student's t test; *p < 0.05

Szabó, R., Sebestyén, M. et al. *ACS Combinatorial Science* 19: 246-254(2017)

Mechanism of uptake of the CF labeled polypeptides in the presence of endocytosis inhibitors

Summary

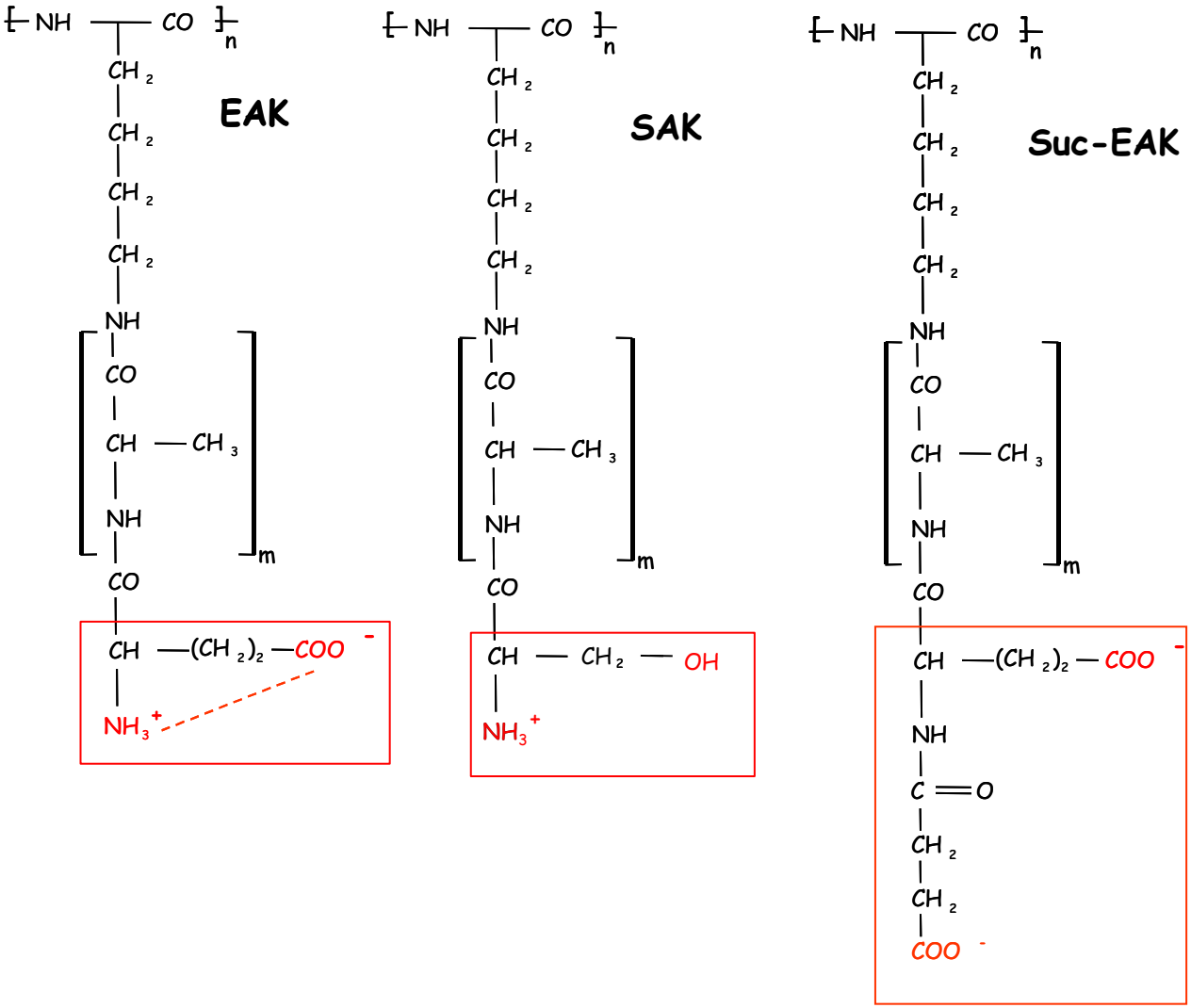
Compound	Colchicin[4]		EIPA[1,2,3]		Cytochalasin D[1,2]		Metil-β-ciklodextrin	
	HT-29	HepG2	HT-29	HepG2	HT-29	HepG2	HT-29	HepG2
Poly[Lys]	-	-	-	-	-	-	+	-
H_iK	-	-	-	+	+	+	+	+
AK	-	-	-	-	+	-	-	-
ALK	-	-	-	-	+	-	+	-

HepG2 Pinocytosis (poly[Lys], H_iK, AK, ALK)
Macropinocytosis (H_iK)
Lipid raft/caveola (H_iK)

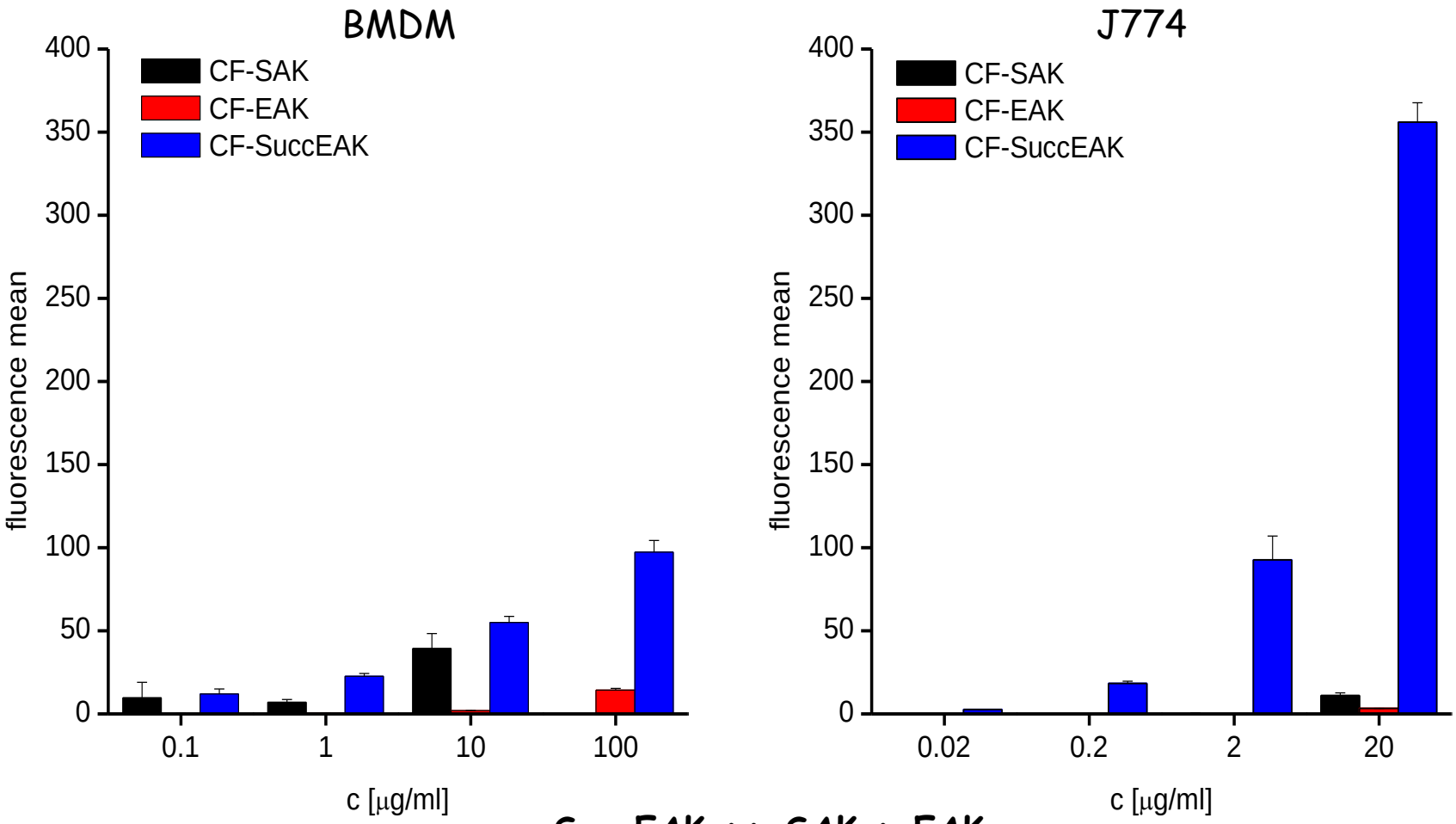
HT-29 Pinocytosis (poly[Lys], H_iK, AK, ALK)
Macropinocytosis (poly[Lys], H_iK, ALK)
Lipid raft/caveola (poly[Lys], H_iK, ALK)

[1]Nakase, I. et al, Mol. Ther. (2004) 10: 1011-1022
[2]Delwig, A. et al, Arthr. Res Ther. (2006) 312: 1345-1360
[3]Heikkilä, O. et al, J. Virol. (2010) 84: 3666-3681
[4]Piasek, A. et al, hematol. Blood Transf. (1985) 29: 511-513
[5]Rodal, S.K. et al, Mol. Biol. Cell (1999) 10: 961-974

The structure of polypeptides



Uptake of polycationic, amphotheric and polyanionic polypeptides



SuccEAK >> SAK > EAK

Conclusions

1. Branched polypeptides are taken up by J774 cells. This process is time and concentration dependent.

2. The structure (e.g. charge properties) influence greatly the uptake. The following order could be established: polycationic >> polyanionic >> amphoteric polypeptides.

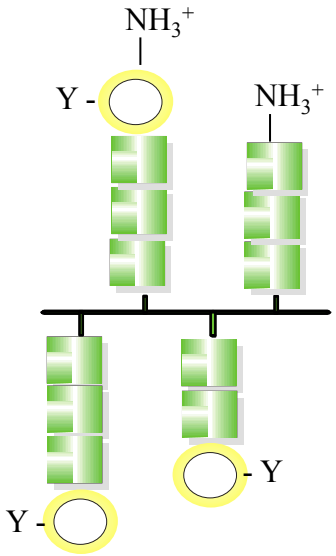
3. Polypeptides could be detected in the cytoplasm.

4. Higher level of uptake was observed by fixed than living cells.

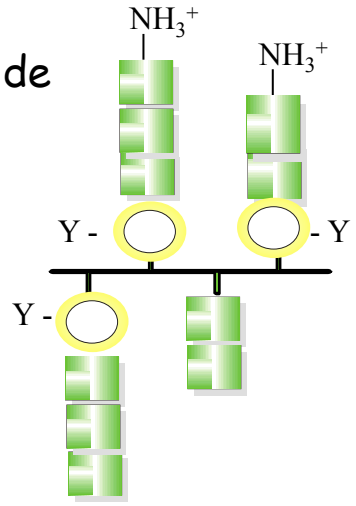
Conclusion

Solution conformation,
cytotoxicity and
cellular uptake
of the XAK/AXK type branched polypeptide
are influenced by the

- identity, character (hydrophobic, hydrophilic), charge
- position in the side the side chain
- number of amino acid X.



XAK, poli[Lys(X_i-DL-Ala_m)]

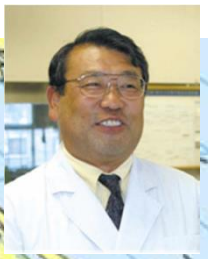


AXK, poli[Lys(DL-Ala_m-X_i)]

Thanks a lot!

Partners around...

Cooperation - multidisciplinarity



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Saclay

Budapest

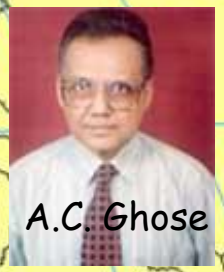
Barcelona

Palermo

Tokyo

Kyoto

Kumamoto



Kolkata

Acknowledgements

OTKA

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UNIVERSITAS BUDAPESTINENSIS DE ROLANDO EÖTVÖS NOMINATA

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NKTH

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ANR
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EACR

BÉRES
GYÓGYSZERGYÁR RT.