INSTITUTE OF MACROMOLECULAR CHEMISTRY

Prague
Czech Republic

Polysaccharide-*graft*-poly(2-alkyl-2-oxazoline) hybrid copolymers: Versatile materials for bioapplications

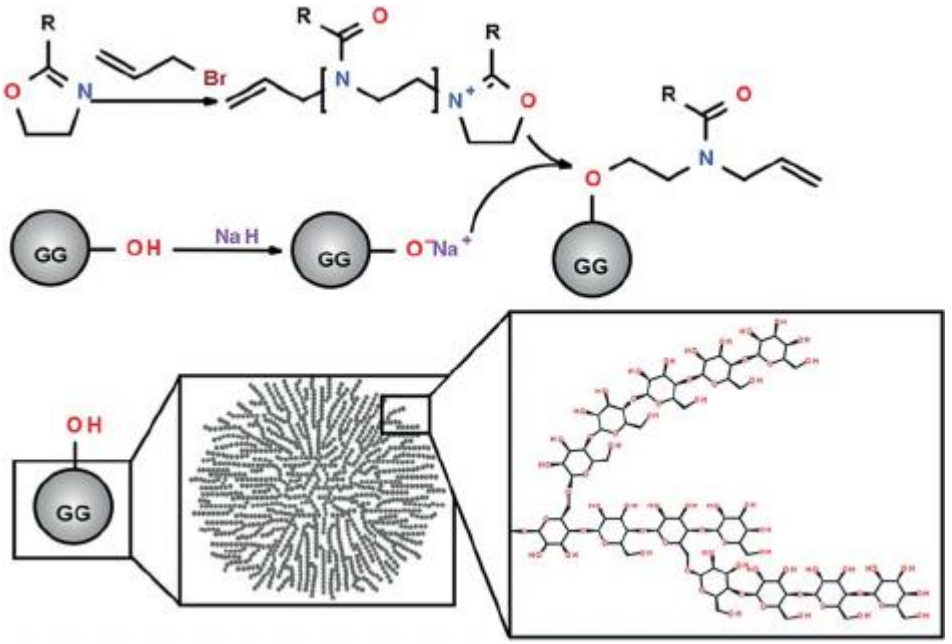
Martin Hrubý, Lenka Loukotová, Mariia Rabyk

cost
EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY

1st CA17140 COST CONFERENCE, 15th-17th October 2019, Riga, Latvia

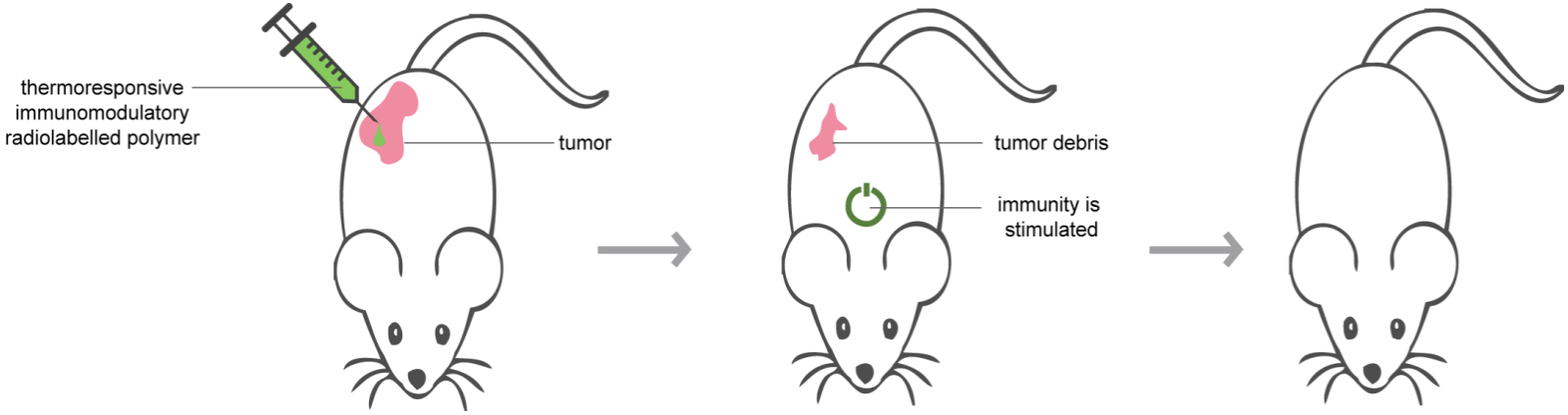
Polysaccharide-*graft*-poly(2-alkyl-2-oxazolines)

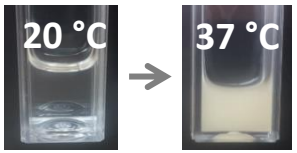
- Poly(2-alkyl-2-oxazoline) part:
  - Synthetic
  - Stimuli-responsive
  - Easy functionalization
- Polysaccharide part:
  - Natural
  - Biologically active
  - Biodegradable



Immunoradiotherapy

- a **conceptually new cancer** treatment
- a possible **synergistic effect** of both immunotherapy and radiotherapy
- **treatment principle**:



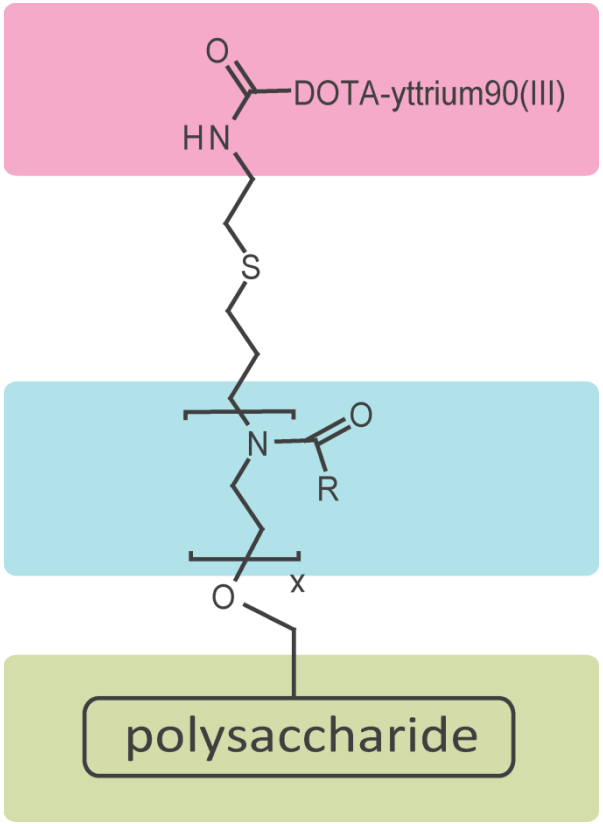


i. polymer forms **a depot** at the injection site - **thermoresponsivity**

ii. **radiation** kills tumor cells (including cells protecting tumor from immune response)

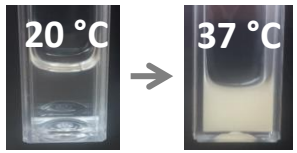
iii. **immunomodulator** enhances following immune response against tumor cell debris and metastasis

Immunoradiotherapy – polymer drug design



– therapeutic radiation – yttrium-90(III)

β^- decay, $T_{1/2}$ = 64.1 h



– thermoresponsiveness

poly(2-isopropyl-2-oxazoline-*co*-2-butyl-2-oxazoline)

biocompatibility, radioresistivity[1]

– immunomodulatory polysaccharide

non-specific immunity activation, biodegradable

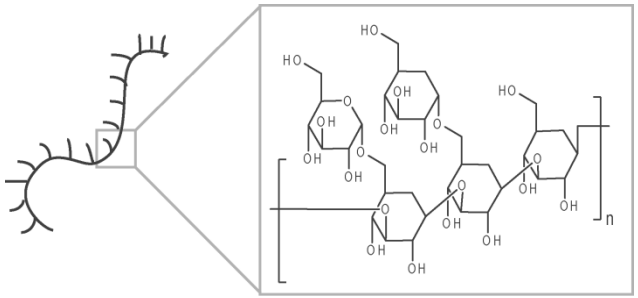
[1] O. Sedlacek *et al.*, Thermoresponsive polymers for nuclear medicine: Which polymer is the best?, *Langmuir*, **2016**, *32*, 6115–6122.

Immunoradiotherapy – polymer drug design

Used immunomodulatory polysaccharides

- **β-glucan from *Auricularia auricula-judae***

  - **anticancer** and immunostimulatory properties[1]



Auricularia auricula-judae

[1] M. Zhaocheng *et al.*, Structure and chain conformation of β-glucan isolated from *Auricularia auricula-judae*, *Biopolymers*, **2008**, *89*, 614–622.

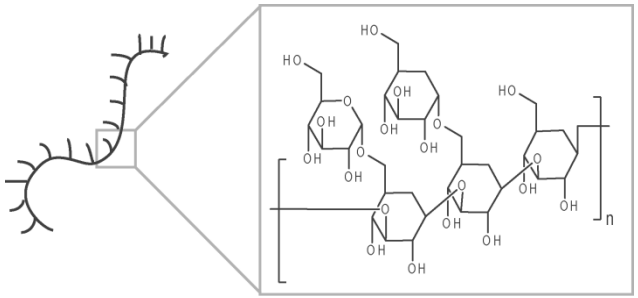
Immunoradiotherapy – polymer drug design

Used immunomodulatory polysaccharides

- **β-glucan from *Auricularia auricula-judae***

  - **anticancer** and immunostimulatory properties[1]

Auricularia auricula-judae

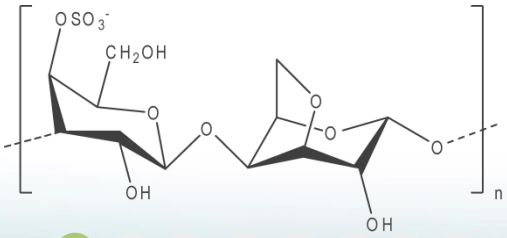


- **κ-carrageenan from *Kappaphycus alvarezii***

  - **anticancer** and immunostimulatory properties[2]

  - agent for the induction of **experimental inflammation** and edema[3]

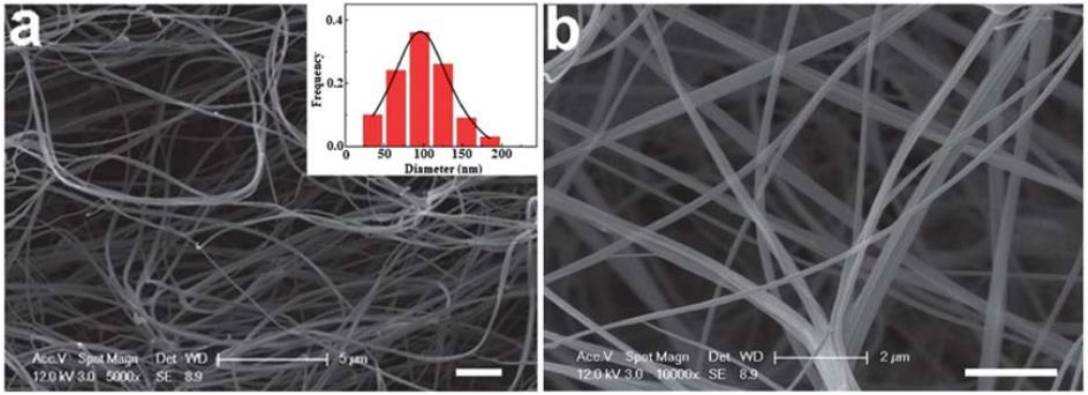
Kappaphycus alvarezii



[1] M. Zhaocheng *et al.*, Structure and chain conformation of β-glucan isolated from *Auricularia auricula-judae*, *Biopolymers*, **2008**, *89*, 614–622.
[2] M. Raman *et al.*, κ-Carrageenan from marine red algae, *Kappaphycus alvarezii* – A functional food to prevent colon carcinogenesis. *J. Funct. Foods*, **2015**, *15*, 354–364.
[3] D. Salvemini *et al.*, Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation, *British journal of pharmacology*, **1996**, *11*, 829–838.

Structural aspects of the polysaccharides I

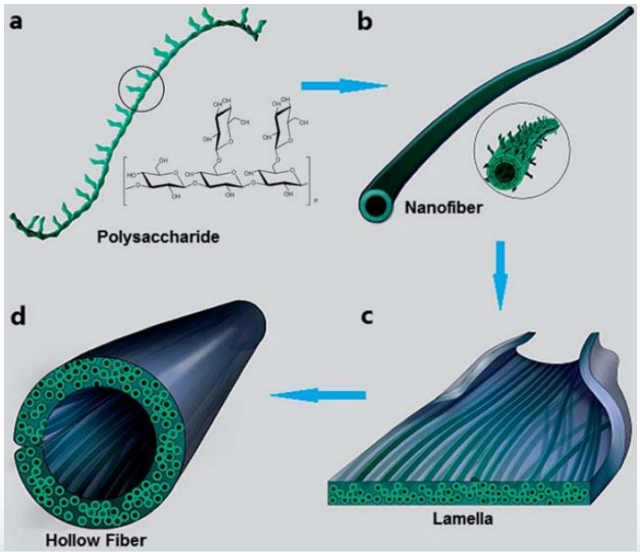
β-Glucan – forms hollow nanofibres in water by self-assembly[1]



SEM: Distribution of sizes

SEM: Morphology

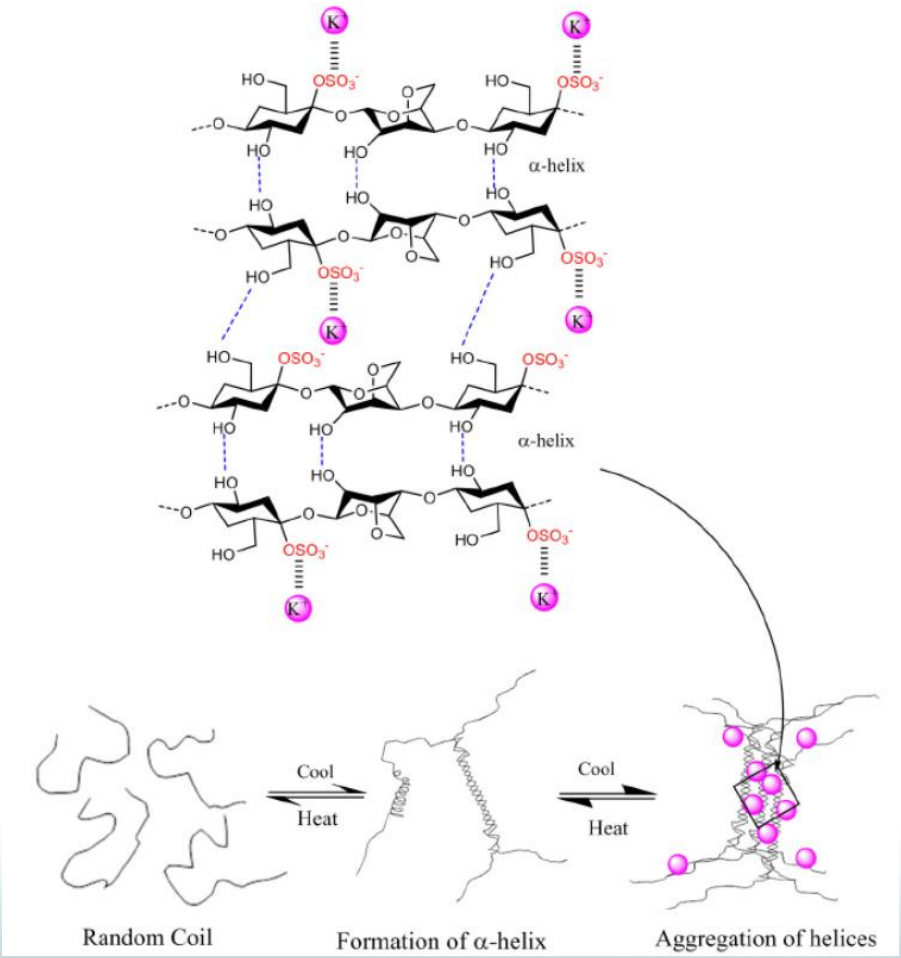
Hollow nanofibers formation



[1] S. Xu *et al.*, Construction of high strength hollow fibers by self-assembly of a stiff polysaccharide with short branches in water, *J. Mater. Chem. A*, 2013, 1, 4198–4206.

Structural aspects of the polysaccharides II

κ-Carrageenan – thermoresponsive (UCST), K^+ and Ca^{2+}-responsive



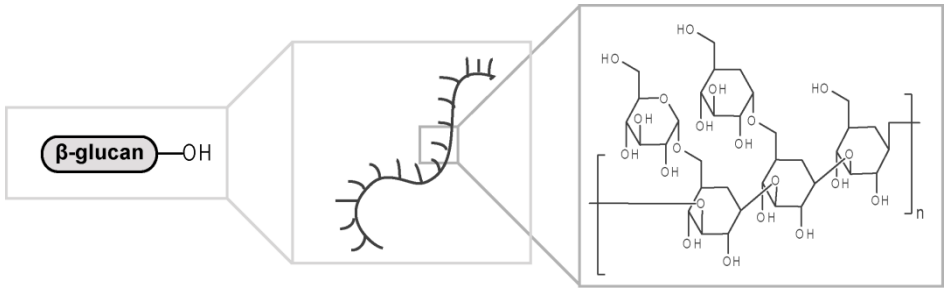
[1] N. Rein-Knudsen *et al.*, Seaweed Hydrocolloid Production: An Update on Enzyme Assisted Extraction and Modification Technologies, *Mar. Drugs* 2015, 13, 3340-3359.

A. Polymer synthesis and characterization

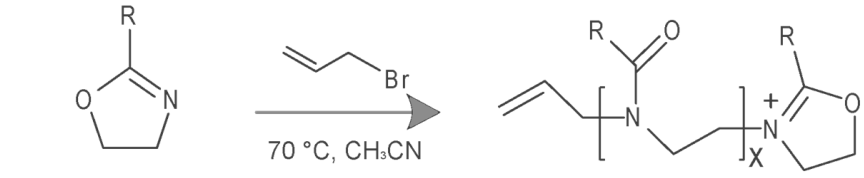
β-Glucan from *Auricularia auricula-judae*

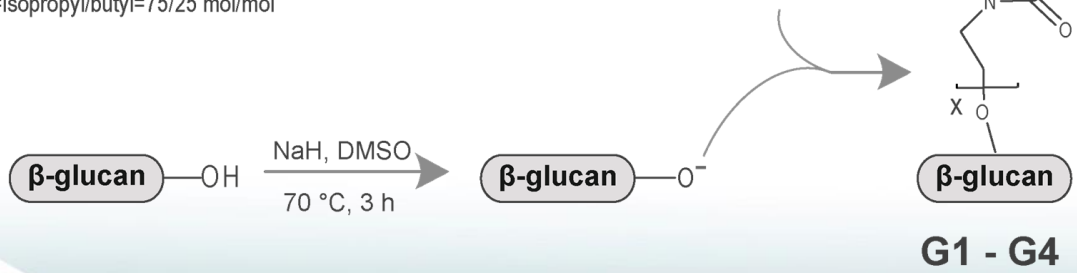
Auricularia auricula-judae

1. **Extraction** of **β-glucan** from *Auricularia auricula-judae*



2. **Grafting** of β-glucan with **poly(2-alkyl-2-oxazoline)s** (POX)





Sample	Theor. graft length (Da)
G1	500
G2	1000
G3	2500
G4	5000

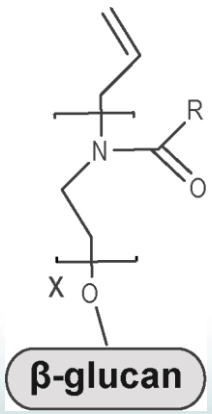
β-Glucan from *Auricularia auricula-judae*

Auricularia auricula-judae

3. Polymer characterization

Sample	Theor. graft length (Da)	Found graft length (Da)[1]	POX content (wt. %)[2]	M_w (Da) [1]	Glucose units per graft
G1	500	590	26	3.7×10^6	9
G2	1000	1140	47	6.4×10^6	7
G3	2500	2290	70	7.5×10^6	6
G4	5000	4180	80	1.6×10^7	6

[1] Determined by SEC-MALS.
[2] Determined by elemental analysis.

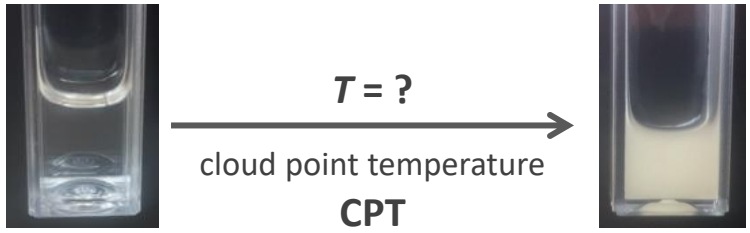


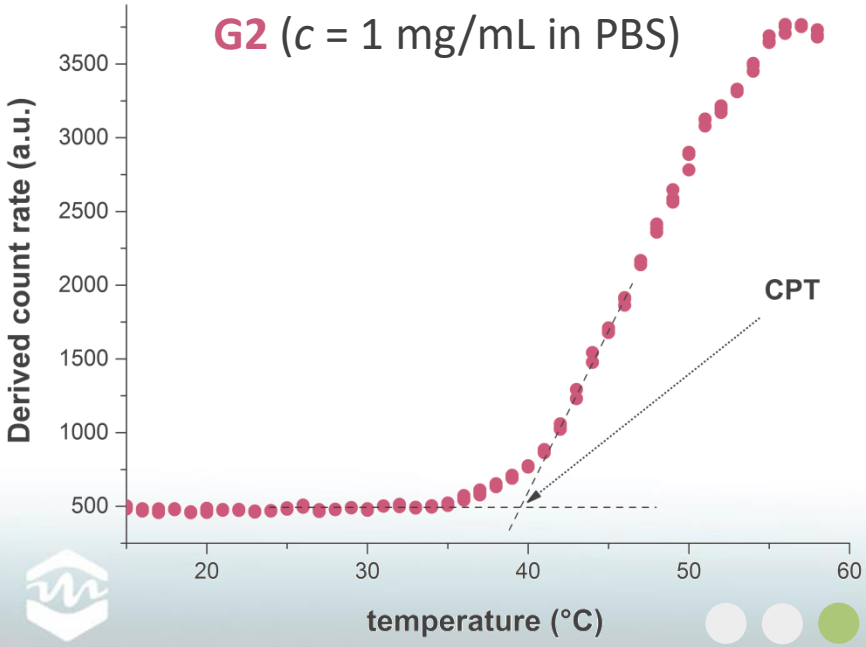
β-Glucan from *Auricularia auricula-judae*

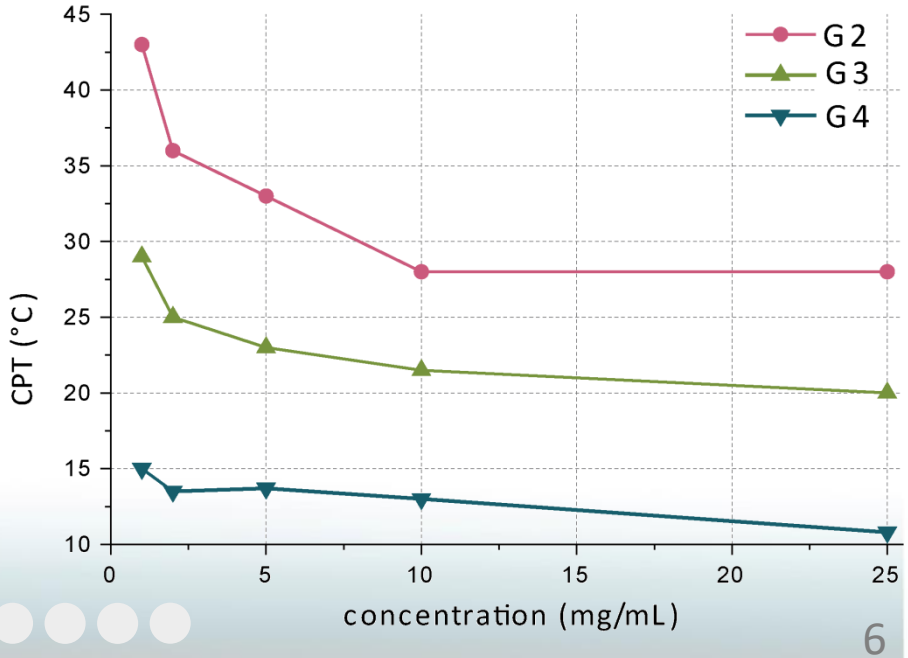
Auricularia auricula-judae

4. Dynamic light scattering – study of **temperature-dependent** behavior

Sample	Theor. graft length (Da)
G1	500
G2	1000
G3	2500
G4	5000







β-Glucan from *Auricularia auricula-judae*

Auricularia auricula-judae

4. Dynamic light scattering – study of **temperature-dependent** behavior

Sample	Theor. graft length (Da)
G1	500
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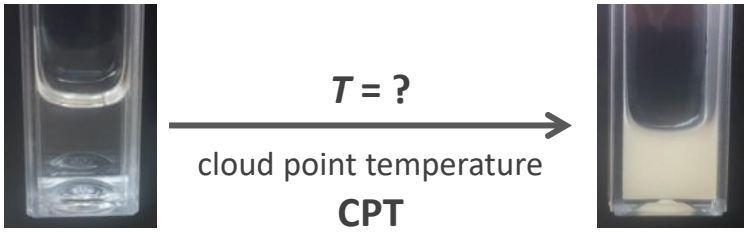
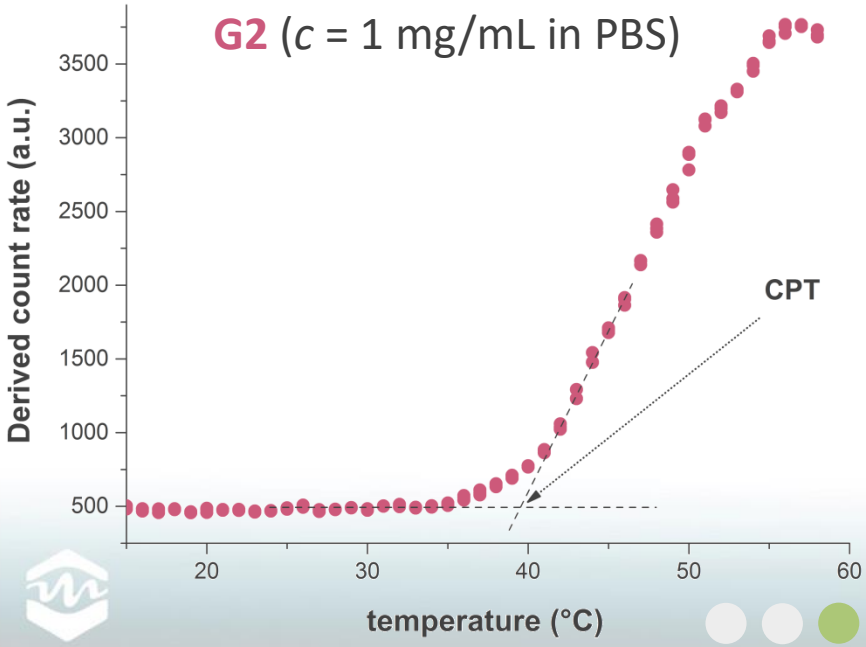
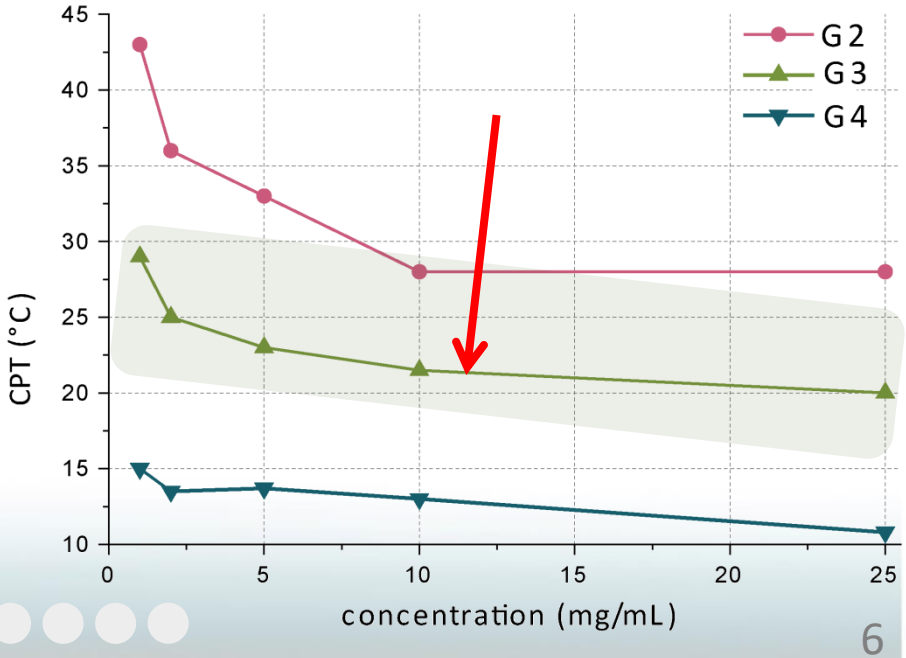






β-Glucan from *Auricularia auricula-judae*

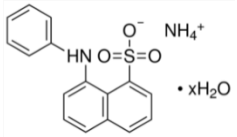
Auricularia auricula-judae

5. Fluorescence measurement

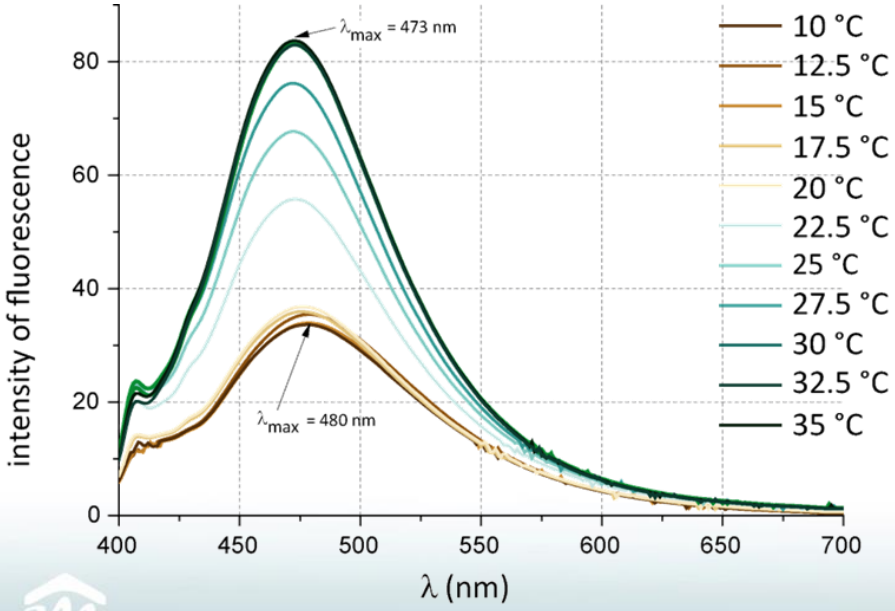
– study of microenvironment **hydrophobicity** during **phase transition**

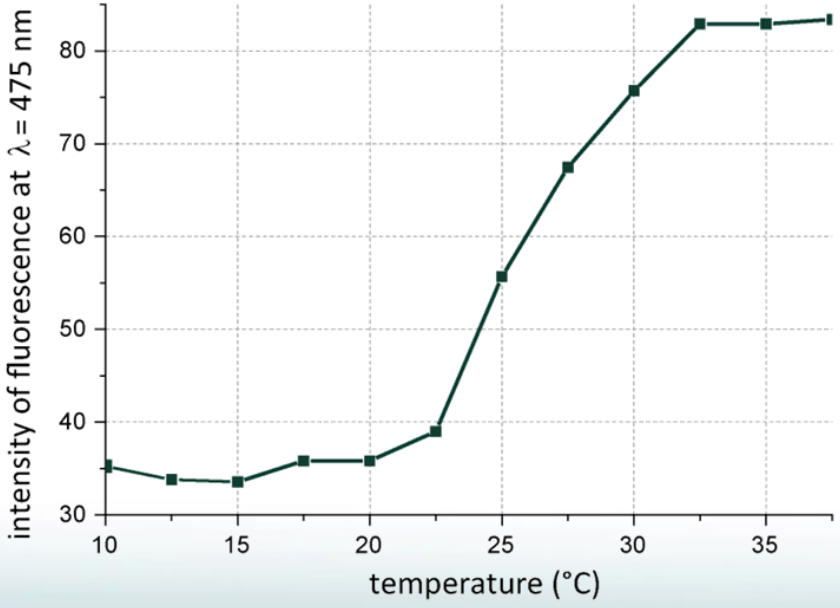
– **aggregation-induced emission** – **fluorescence probe**

(highly fluorescent in aggregated state)



G3 (*c* = 1 mg/mL in PBS), 8-anilino-1-naphthalenesulfonic acid ammonium salt (*c* = 0.25 µmol/mL)

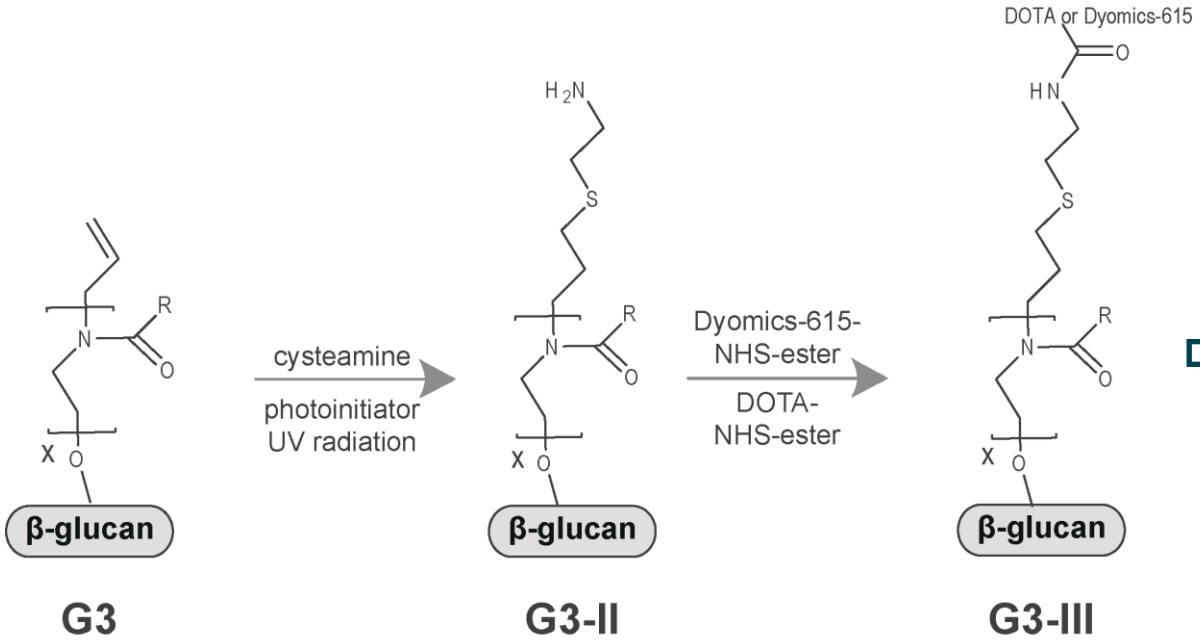




β-Glucan from *Auricularia auricula-judae*

Auricularia auricula-judae

6. Polymer modification



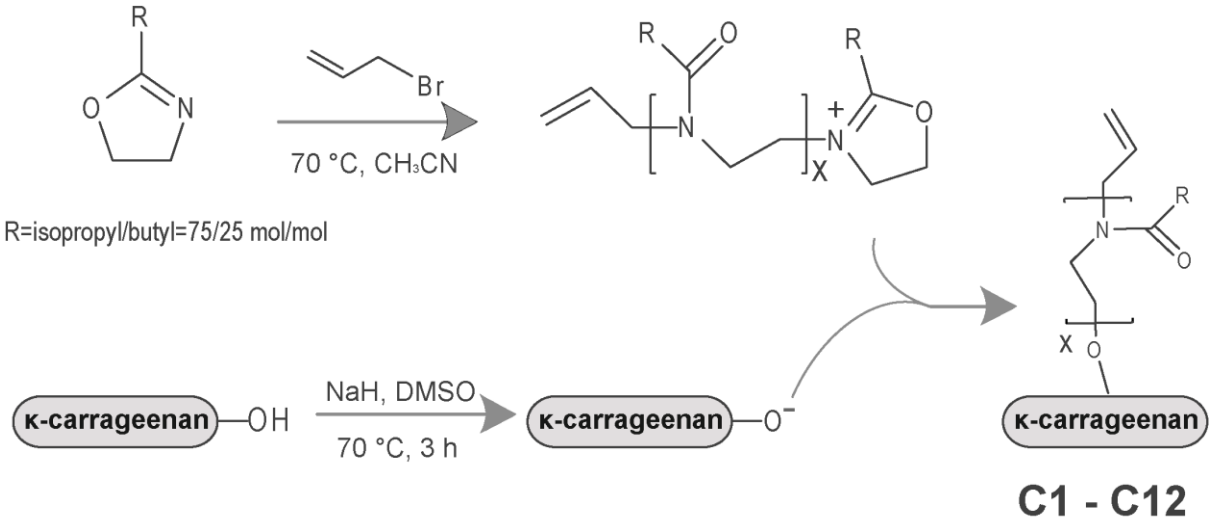
G3 G3-II G3-III

in vitro **experiments**

κ-Carrageenan from *Kappaphycus alvarezii*

1. Grafting of κ-carrageenan with **poly(2-alkyl-2-oxazoline)s** (POX)

Kappaphycus alvarezii

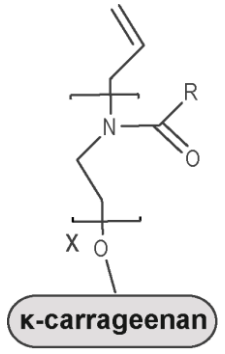


κ-Carrageenan from *Kappaphycus alvarezii*

Kappaphycus alvarezii

2. Polymer characterization

	Theor. graft length (Da)	Found graft length (Da)[1]	POX content (wt. %)[2]	Carbohydrate monomeric units per graft	M_w (Da)[1]
C1	**1000**	**860**	34	9.3	2.3×10^6
C2			26	13.5	1.7×10^6
C3			18	21.8	2.0×10^6
C4			8	55.0	1.0×10^6
C5	**2500**	**1950**	65	5.9	4.5×10^6
C6			60	7.2	2.0×10^6
C7			35	20.5	1.2×10^6
C8			15	59.6	9.5×10^5
C9	**5000**	**4350**	81	5.6	6.3×10^6
C10			74	8.3	5.4×10^6
C11			28	60.8	9.1×10^5
C12			15	132.1	5.3×10^5



C1 - C12

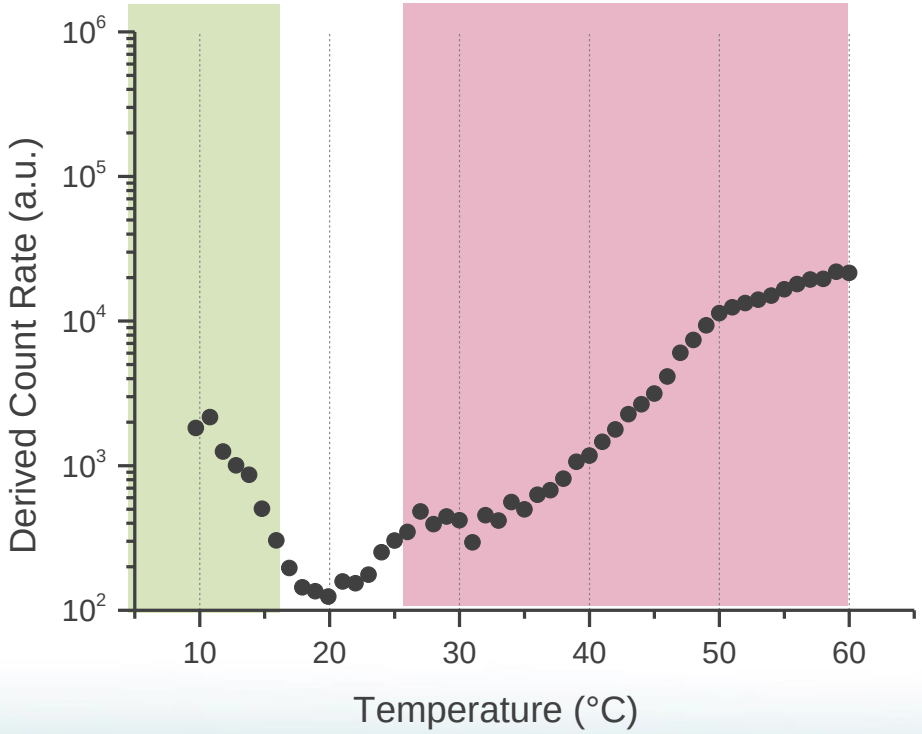
[1] Determined by SEC-MALS.
[2] Determined by elemental analysis.

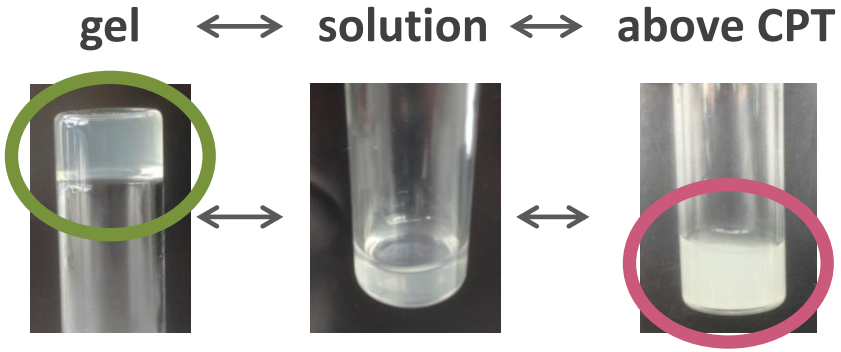
κ-Carrageenan from *Kappaphycus alvarezii*

3. Dynamic light scattering – study of **temperature-dependent** behavior

Kappaphycus alvarezii

C6 (c = 1 mg/mL in PBS), M_n(grafts) = 1950 Da



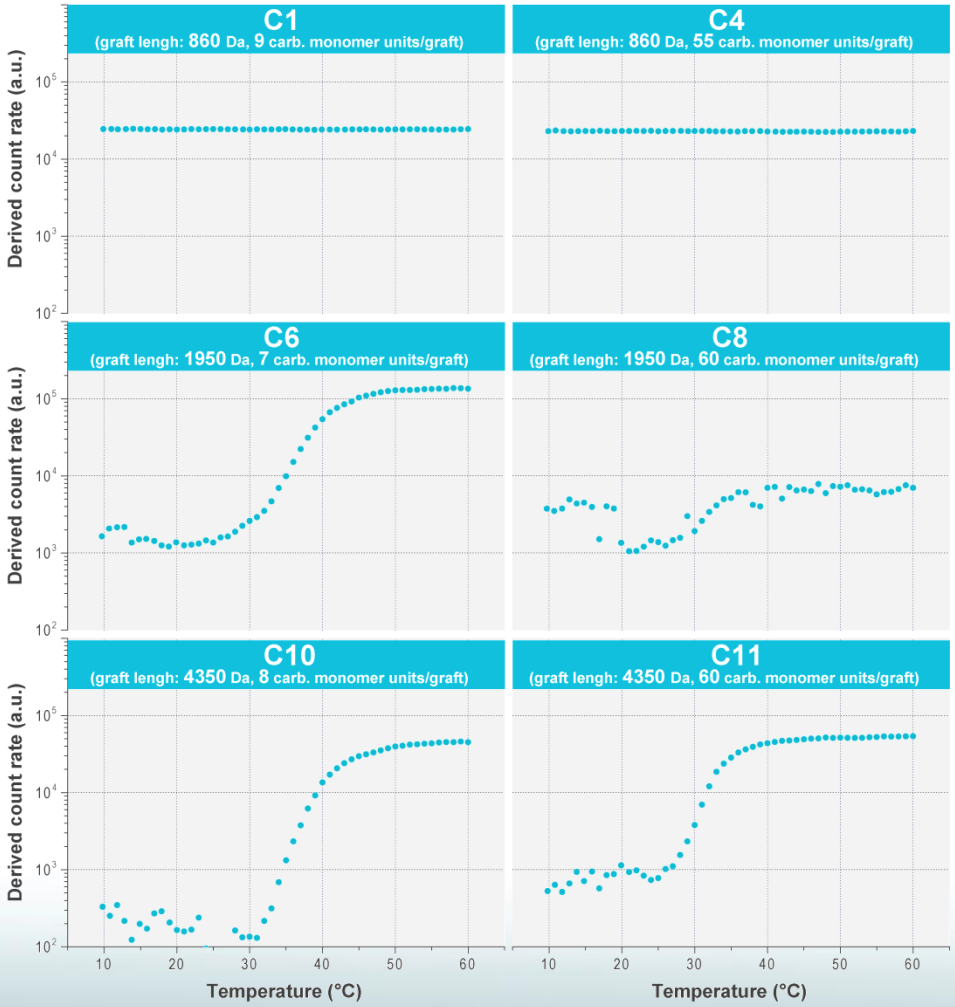


κ-Carrageenan from *Kappaphycus alvarezii*

Kappaphycus alvarezii

3. Dynamic light scattering

increasing grafting density



increasing graft lenght

(*c* = 1 mg/mL in 0.15M NaCl)

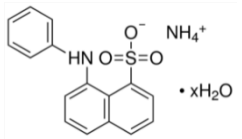
κ-Carrageenan from *Kappaphycus alvarezii*

Kappaphycus alvarezii

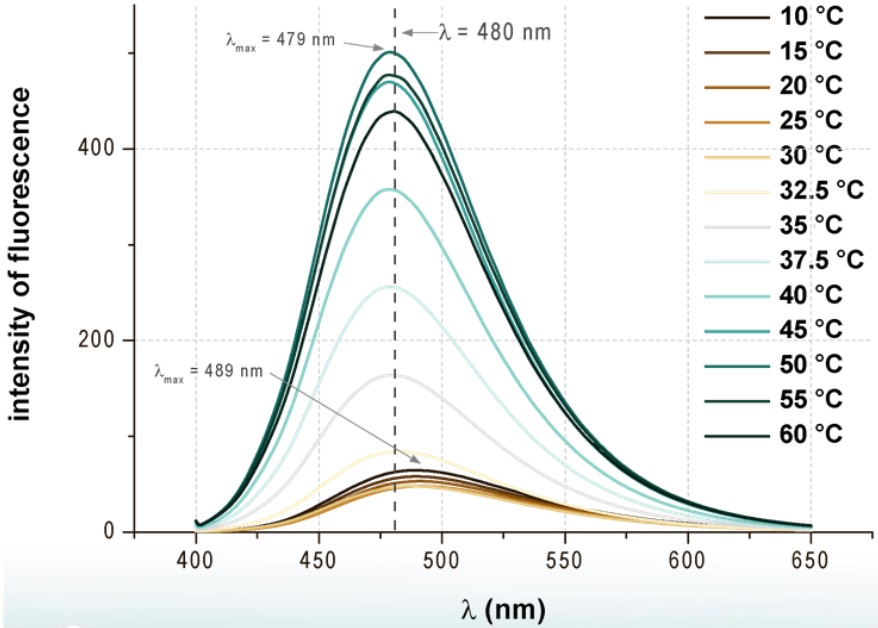
4. Fluorescence measurement

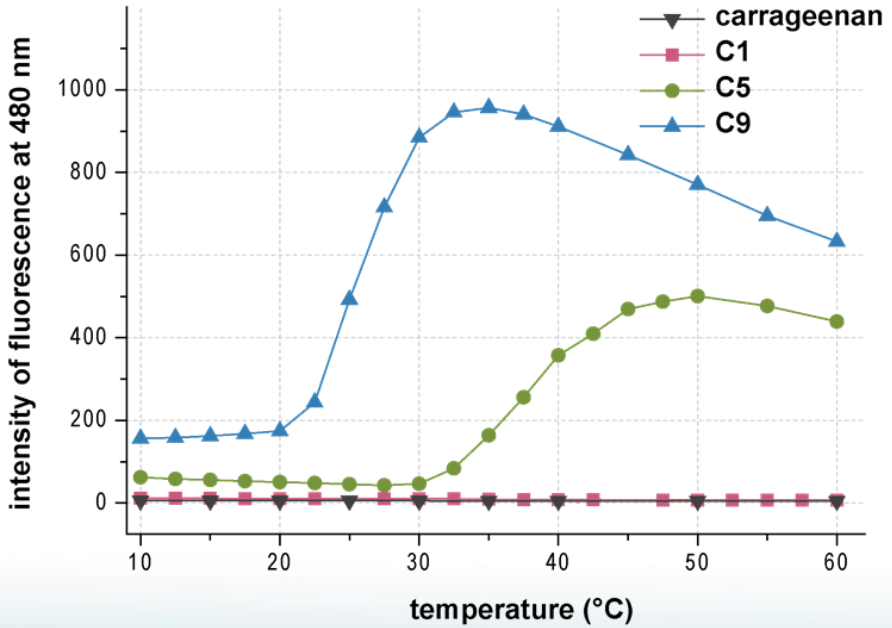
– study of microenvironment **hydrophobicity** during **phase transition**

– **aggregation-induced emission** – **fluorescence probe**



C1, C5 and C9 (*c* = 2.5 mg/mL in NaCl), 8-anilino-1-naphthalenesulfonic acid ammonium salt (*c* = 0.25 µmol/mL)





κ-Carrageenan from *Kappaphycus alvarezii*

5. NMR study – study of **temperature-dependent** behavior

Kappaphycus alvarezii

$$p = 1 - \{I(T) / [I(T_0) \cdot (T_0 / T)]\}$$

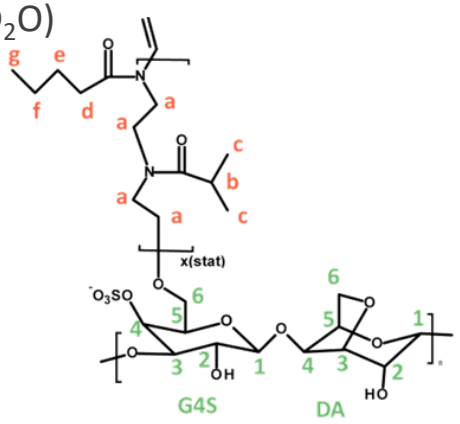
where p is p-fraction, $I(T)$ is the integrated intensity of a signal in the spectrum at the temperature T and $I(T_0)$ is the integrated intensity of the same signal in the case when no phase separation occurs

κ-Carrageenan from *Kappaphycus alvarezii*

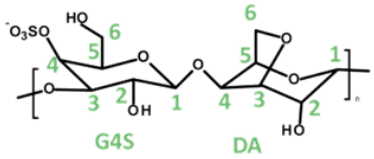
Kappaphycus alvarezii

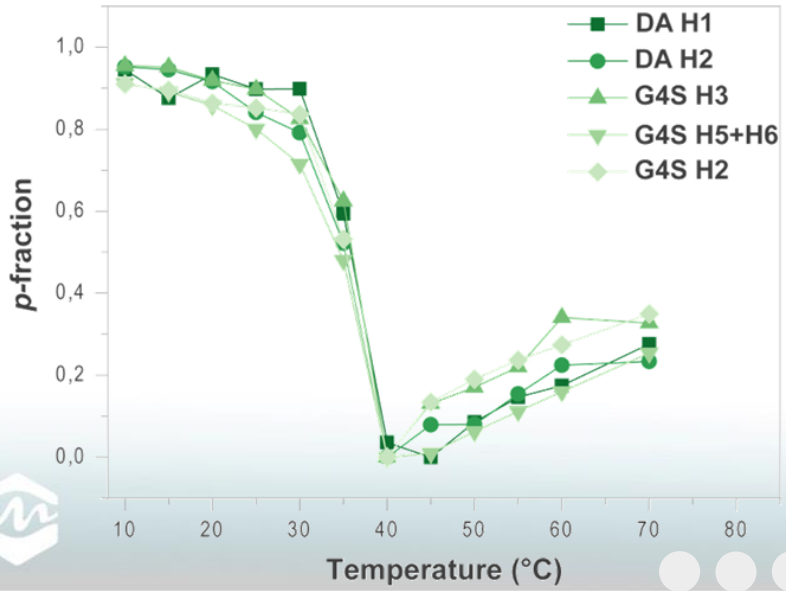
5. NMR study – study of **temperature-dependent** behavior

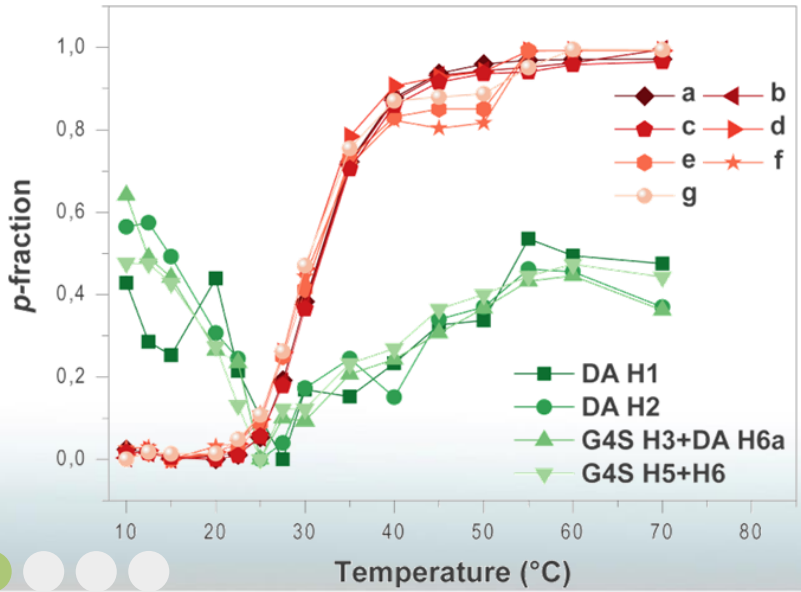
C9 (*c* = 2.5 mg/mL in D_2O)



carrageenan (*c* = 2.5 mg/mL in D_2O)



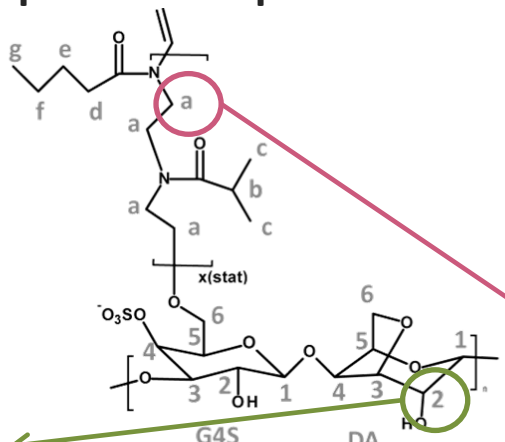




κ-Carrageenan from *Kappaphycus alvarezii*

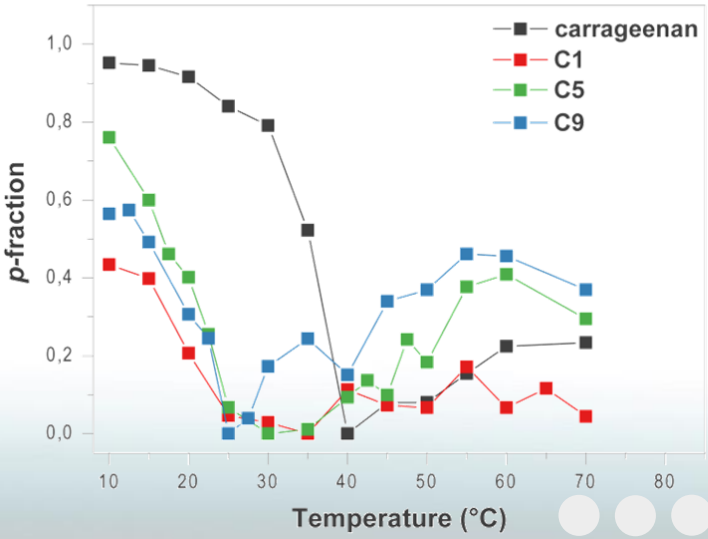
Kappaphycus alvarezii

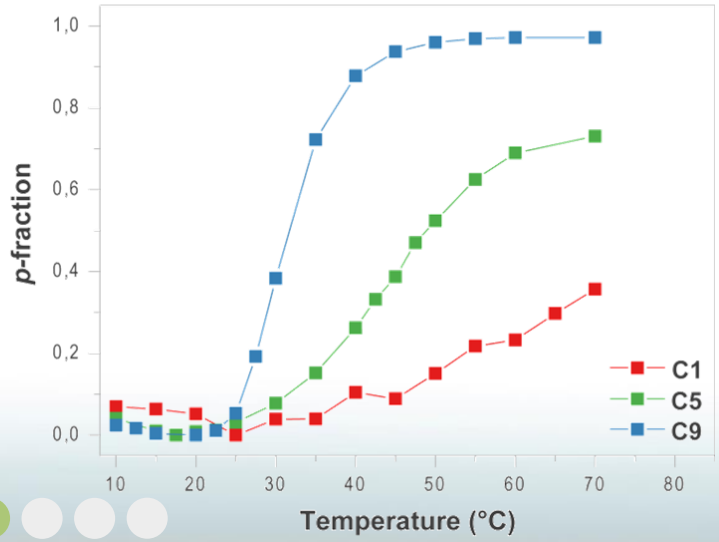
5. NMR study – study of **temperature-dependent** behavior



DAH2 peak

"a" peak





κ-Carrageenan from *Kappaphycus alvarezii*

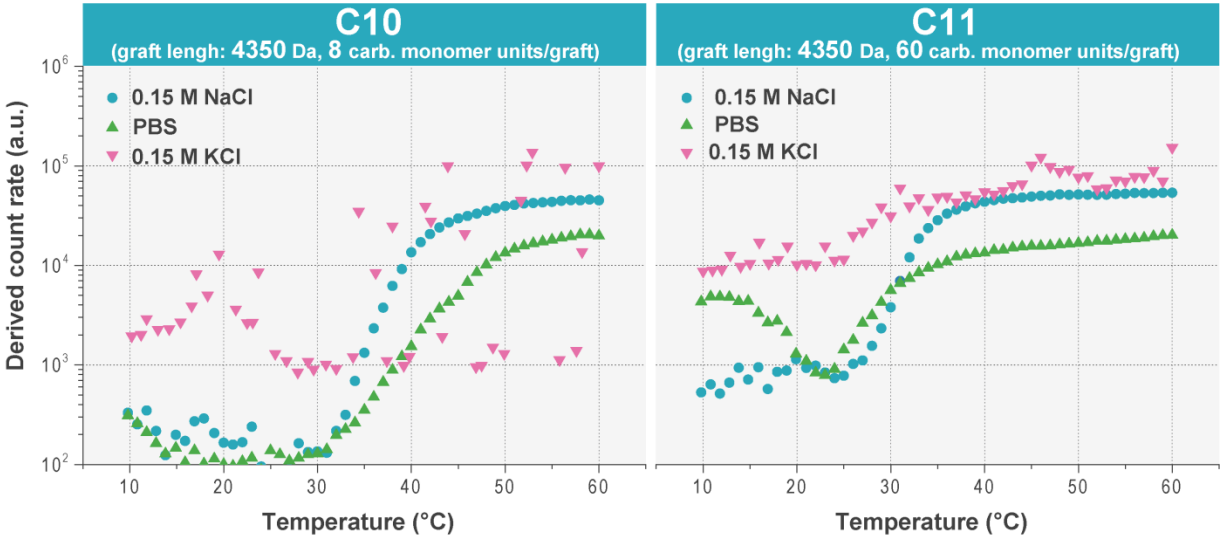
Kappaphycus alvarezii

6. Potassium responsiveness

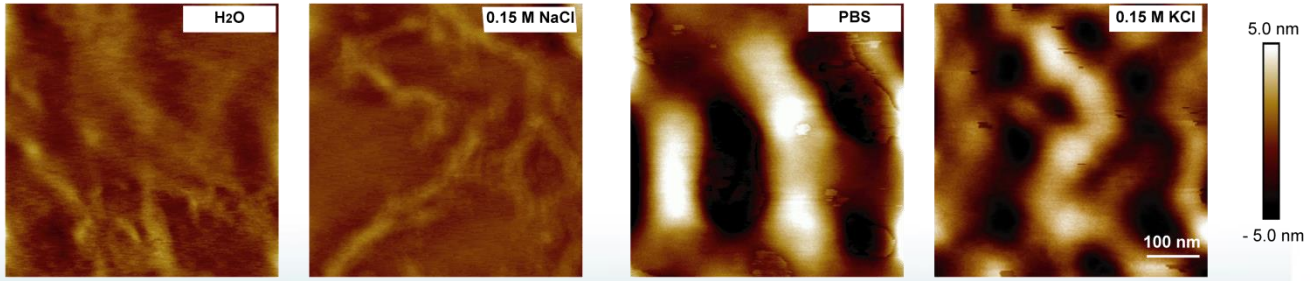
increasing grafting density

- **dynamic light scattering**

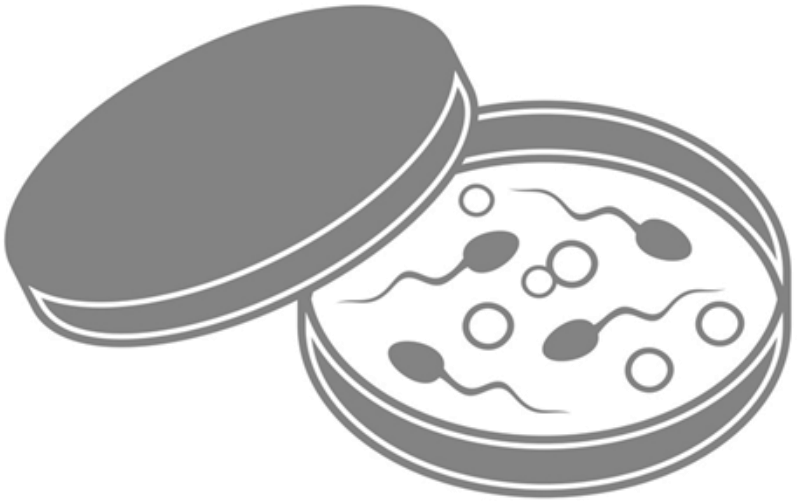
  (*c* = 2.5 mg/mL)



- **atomic force microscopy**



B. *In vitro* experiments



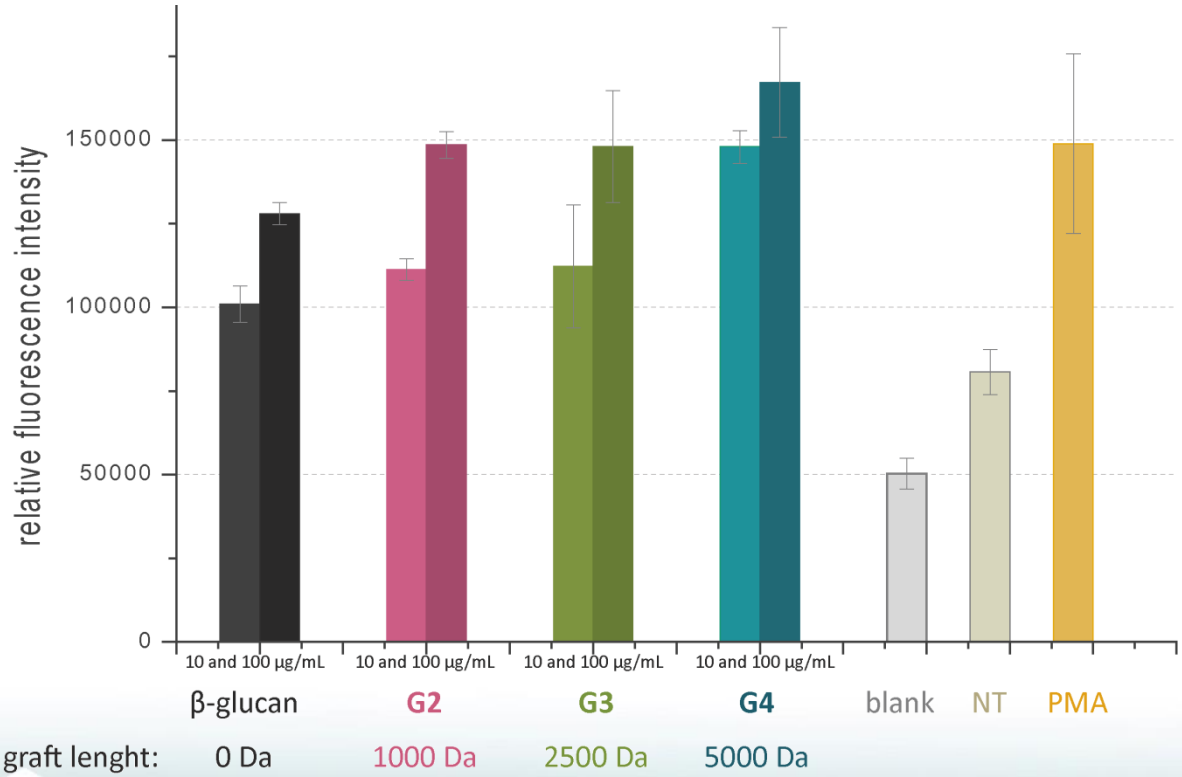
β-Glucan from *Auricularia auricula-judae*

Auricularia auricula-judae

1. Oxidative burst of leukocytes assay

– corresponds to the immunostimulatory activity of polymer

– chemiluminescence reaction of formed hydrogen peroxide with luminol



→ **the oxidative burst response is not influenced by graft length**

β-Glucan from *Auricularia auricula-judae*

Auricularia auricula-judae

1. Oxidative burst of leukocytes assay

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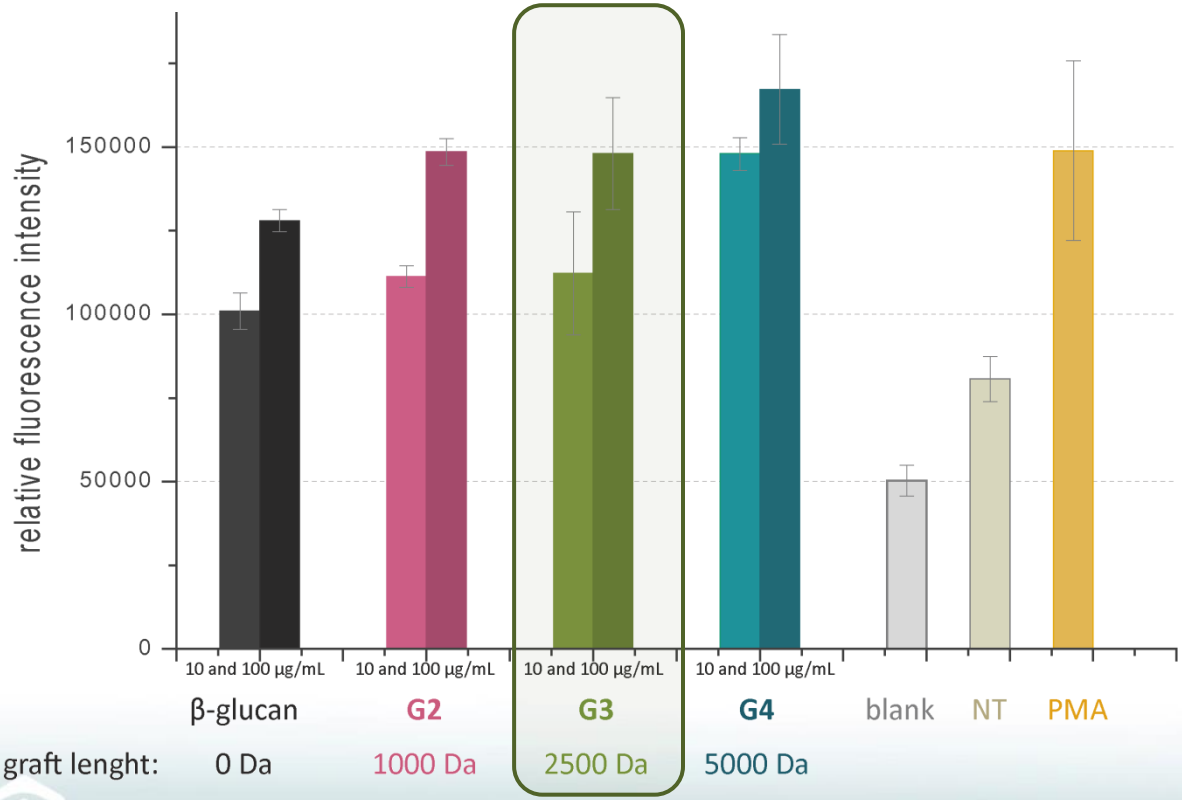

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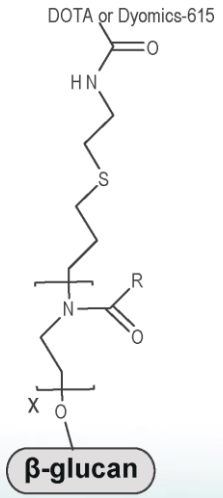


G3-III

β-Glucan from *Auricularia auricula-judae*

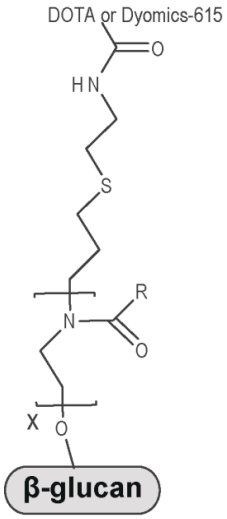
Auricularia auricula-judae

2. Cytotoxicity

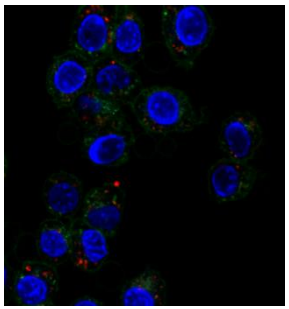
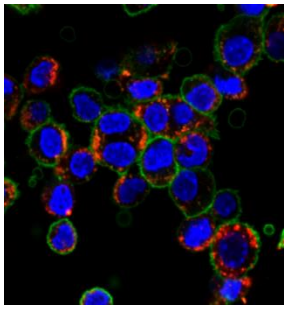
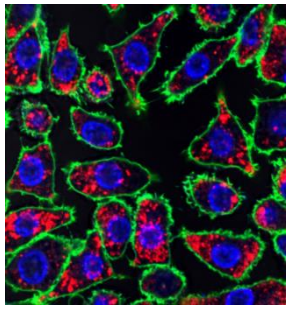
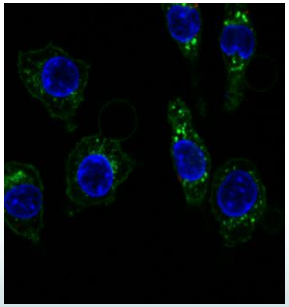
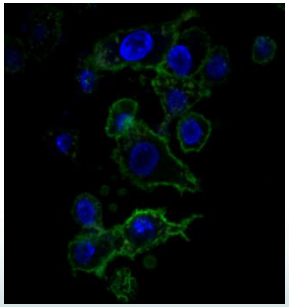
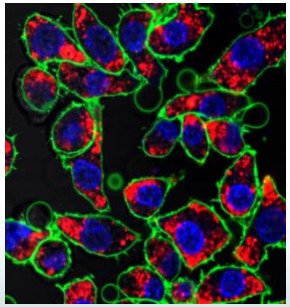
– **negligible** on macrophages RAW 264.7, MCF7 and EL4 cancer cells (AlamarBlue assay)

3. Cellular uptake study using microscopy

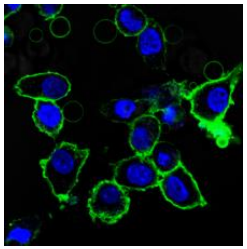
– incubation with RAW cells



G3-III

	15 min	4 h	24 h
G3 grafts 2500 Da			
β-glucan (labelled with Dyomics-615)			

control



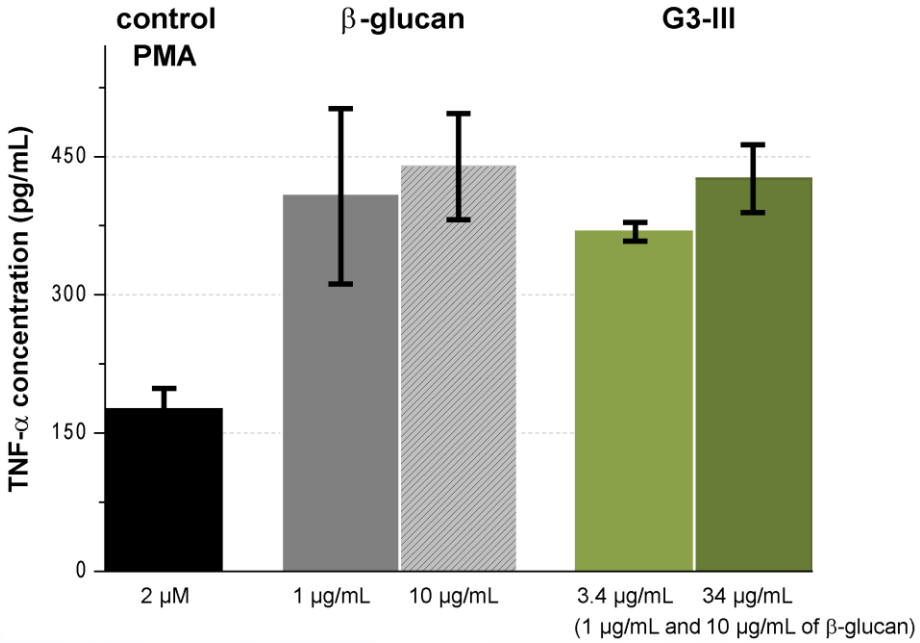
Organelle staining: Green (CellMask Green, cell membrane), blue (Hoechst 33342, nucleus) and red (Dyomics-615 dye, polymers).

β-Glucan from *Auricularia auricula-judae*

Auricularia auricula-judae

4. Tumor necrosis factor α (TNF-α) production

– using an enzyme-linked immunosorbent assay (ELISA) on the leukocytes isolated from human whole blood



→ **optimistic prognosis for cancer treatment**

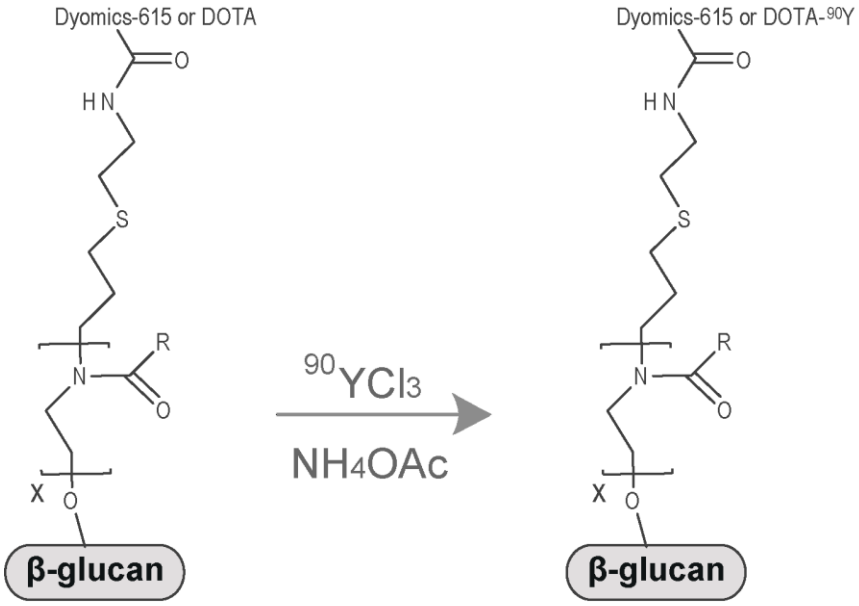
C. Radiolabeling

β-Glucan from *Auricularia auricula-judae*

Auricularia auricula-judae

1. Radiolabeling with yttrium-90(III)

Nuclide	Decay mode	Energy (MeV)	$t_{1/2}$
^{90}Y	β^-	2.28	64 h
^{177}Lu	$\beta^-(\gamma)$	0.497	160 h
^{213}Bi	α, β^-	5.87	46 min
^{225}Ac	α	5.83	10 d
^{223}Ra	α	5.85	11.4 d

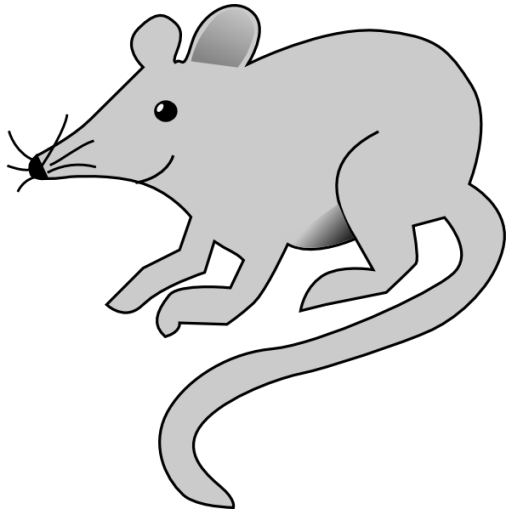


G3-III **G3-III-Y**

***A* = 5 MBq/mg**

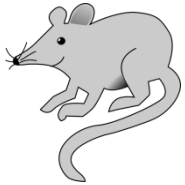
L. Loukotova *et al.*, Thermoresponsive β-glucan-based polymers for bimodal immunoradiotherapy – Are they able to promote the immune system?, *J. Control. Release*, **2017**, *267*, 78 – 91.

D. *In vivo* experiments



β-Glucan from *Auricularia auricula-judae*

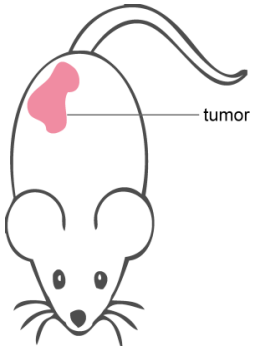
Auricularia auricula-judae



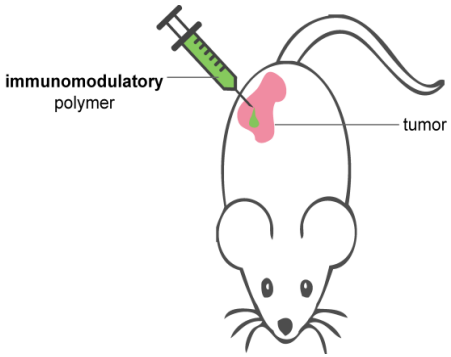
1. Antitumor efficiency

- C57BL/6N mice with murine lymphoma EL4
- groups:

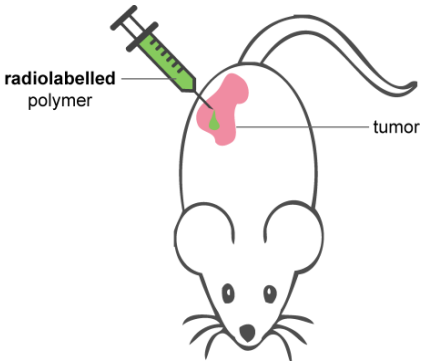
1) Control
- no treatment



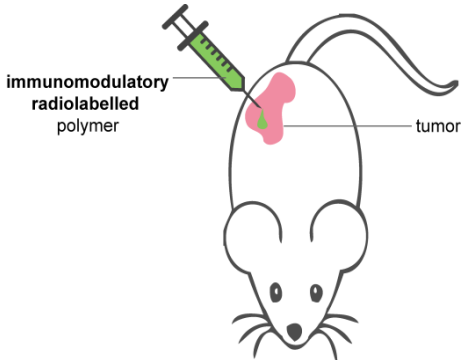
2) IMMUNO
β-glucan-*graft*-POX
G3-III
1 mg/mouse



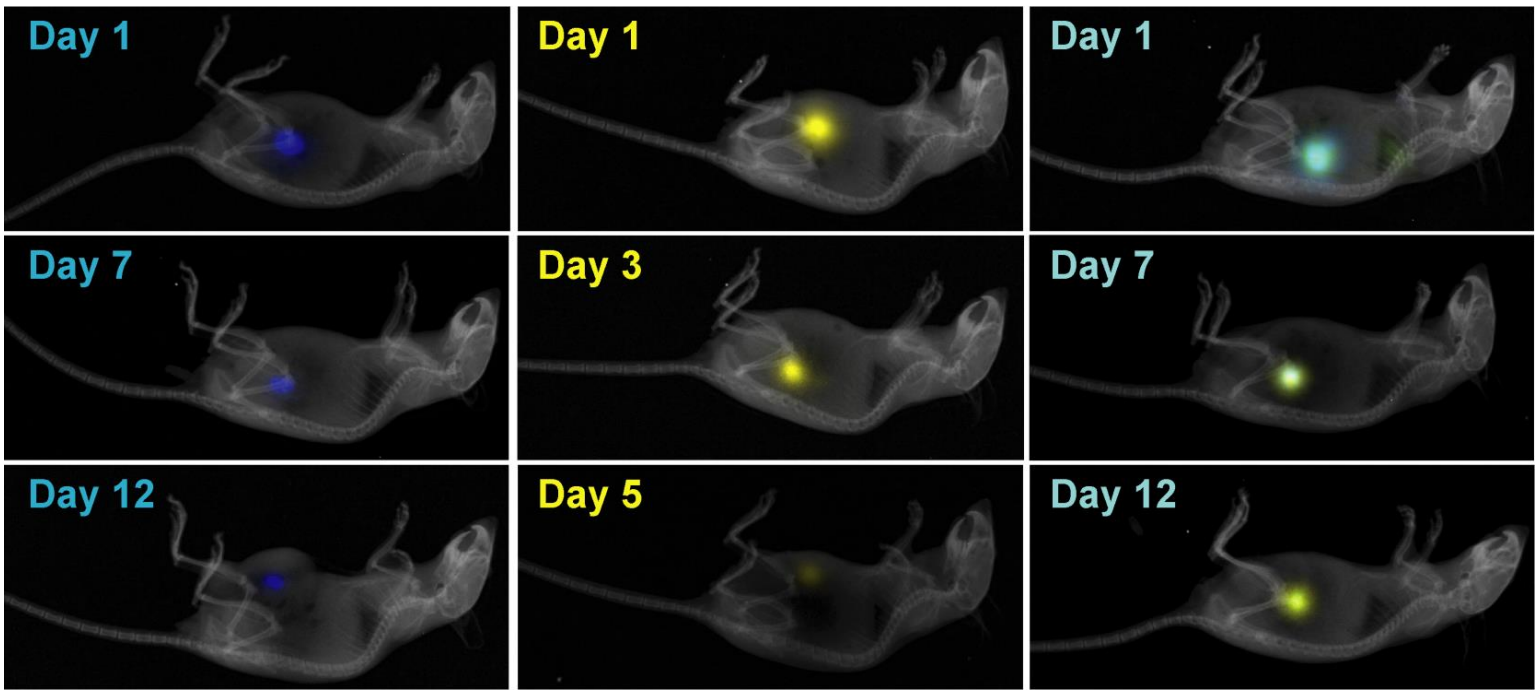
3) RADIO
- POX with ^{90}Y
POX-Y
0.7 mg/4 MBq/mouse



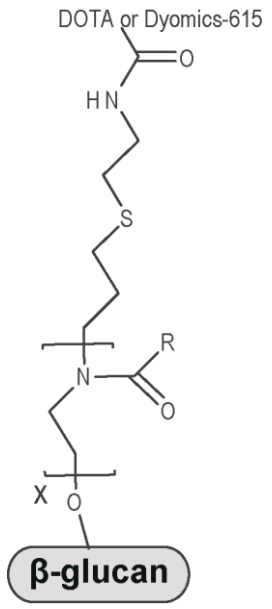
4) IMMUNORADIO
- β-glucan-*graft*-POX with ^{90}Y
G3-III-Y
1 mg/4 MBq/mouse



In vivo imaging



Auricularia auricula-judae

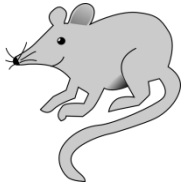


G3-III

Images were done by **composition** of **X-ray images** (**all groups**), **fluorescence images** of the dye **Dyomics-615** (**IMMUNO and IMMUNORADIO**) and **Cherenkov radiation images** of **yttrium-90(III)** **(RADIO and IMMUNORADIO)**

β-Glucan from *Auricularia auricula-judae*

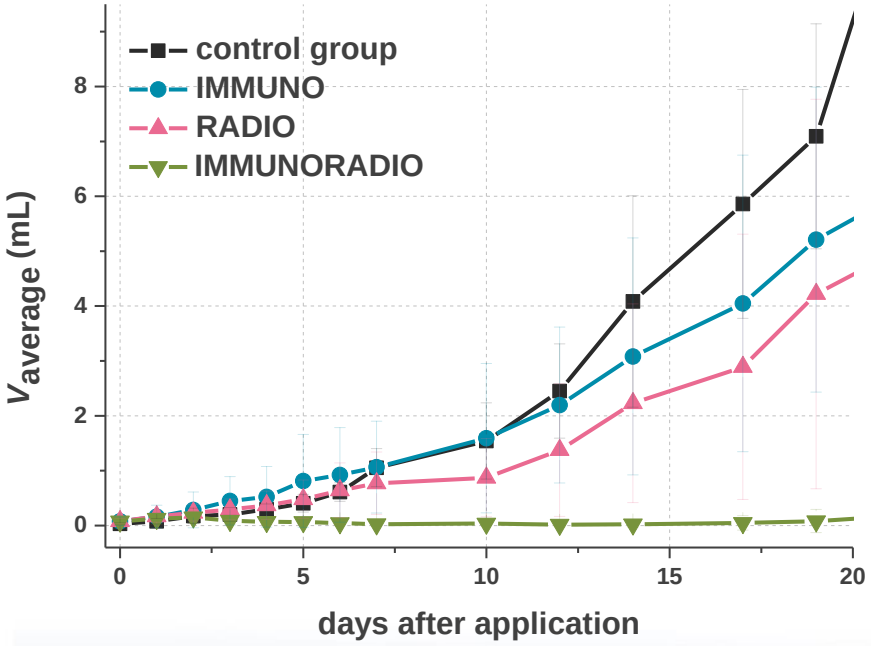
Auricularia auricula-judae



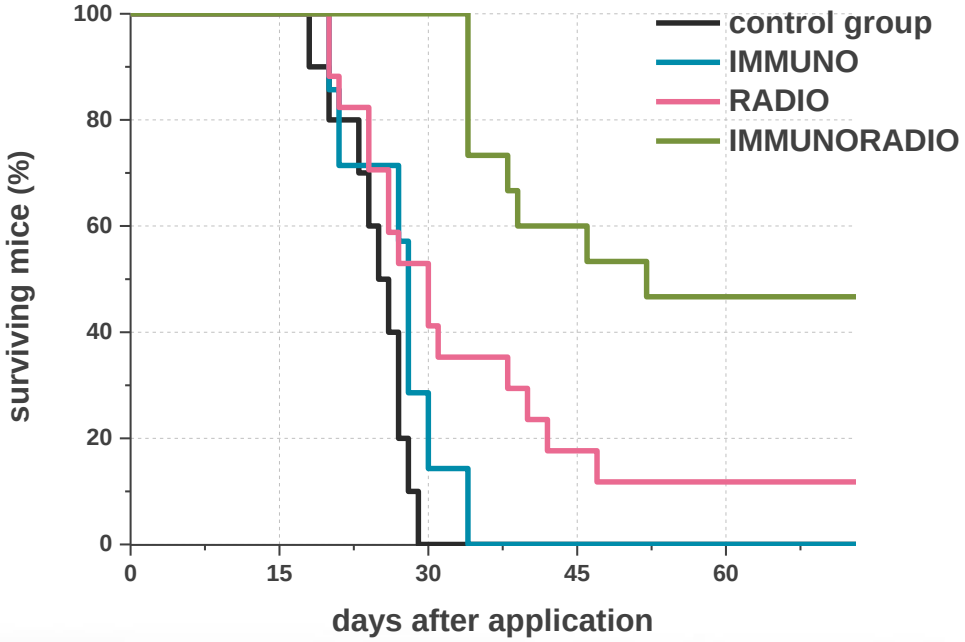
1. Antitumor efficiency

– C57BL/6N mice with murine lymphoma EL4

Primary tumor growth



Survival time



Conclusions

A. Polymer synthesis and characterization

β-glucan-*graft*-POX

- successful **synthesis** of β-glucan-*graft*-POX
- study of **temperature-dependent** behavior using DLS and fluorescence m.
- successful modification of β-glucan-*graft*-POX to bear **DOTA** and **dye** at the graft ends

κ-carrageenan-*graft*-POX

- successful **synthesis** of κ-carrageenan-*graft*-POX
- study of **temperature-dependent** behavior using DLS, fluorescence m. and NMR

B. *In vitro* experiments

- **oxidative burst** of the leukocytes is not influenced by graft length
- negligible **cytotoxicity**, active **cellular uptake**, production of **TNF-α**

C. Radiolabeling

- successful radiolabeling of β-glucan-*graft*-POX with yttrium-90(III)

D. *In vivo* experiments

- **treatment success: 47 % in IMMUNORADIO group**
- observed **synergistic effect** of using IMMUNO- and RADIOtherapy in combination

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CHEMISTRY
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Tomáš Heizer ….

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