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Glycopolyoxazoline-lectin interactions. Effects of ligand structure on clustering kinetics

Summary — Turbidimetric measurements were used to examine the clustering of concanavalin A (Con A) with glucosylated polyoxazolines. For homopolymers, and also block copolymers, precipitation of Con A occured faster with longer glucoslyated chains and higher valency of the ligand. For statistical copolymers, the same trend was observed for increasing spacing between glucose residues or decreasing epitope density. Also the nature of the non-binding side chains appeared to have impact on the clustering kinetics.
Keywords: polyoxazoline, glycopolymer, lectin, clustering kinetics.

ODDZIAŁYWANIA GLIKOPOLIOKSAZOLINA-LEKTYNA: WPŁYW STRUKTURY LIGANDU NA KINETYKĘ POWSTAWANIA KLASTRÓW
Streszczenie — Metodą turbidymetryczną zbadano proces tworzenia się klastrów z udziałem konkanawaliny A (Con A) oraz glikozylowanej polioksazoliny. W odniesieniu do glikolizowanych homopolimerów a także kopolimerów blokowych strącanie klastrów z konkawalaniną A przebiegało szybciej wówczas, gdy glikozylowane łańcuchy były dłuższe a wartościowość ligandów większa. W przypadku glikolizowanych kopolimerów statystycznych takie samo zjawisko zaobserwowano wraz ze wzrostem odległości między resztami glukozowymi w polimerowym łańcuchu oraz zmniejszeniem się gęstości epitopów (miejsc bezpośredniej interakcji). Charakter niewiążących, bocznych łańcuchów makrocząsteczki również wpływa na szybkość tworzenia klastrów.
Słowa kluczowe: polioksazolina, glikopolimer, lektyna, kinetyka tworzenia klastrów.

Many biological processes, such as immune response, fertilization or cell-cell recognition, are mediated by carbohydrate-lectin interactions [1, 2]. Although individual interactions are only weak, enhanced binding of the lectin is enabled by multiple interactions with multivalent carbohydrates, known as the cluster glycoside effect [3]. Synthetic multivalent carbohydrate ligands cross-link, cluster, and ultimately precipitate lectins and are therefore beneficial probes to investigate the underlying mechanisms involved in these biological processes [4].

A great variety of synthetic macromolecular glycoclusters based on, for instance, cyclodextrins [5], dendrimers [6—8] or colloidal particles [9—13] have been designed and used as high-affinity multivalent ligands for lectins [14]. However, the highest binding activities were determined for linear glycopolymers with pendant carbohydrate groups [15]. Linear polymers of defined architecture and narrow molecular weight distribution, prepared by controlled polymerization techniques, were shown to act as well-defined scaffolds for the attachment of carbohydrates [16—18]. Furthermore, these systems offer the possibility to independently control the valency as well as the density and spatial arrangement of the recognition elements.

Glycosylated polyoxazolines have some advantages over other multivalent ligands reported in literature, including facile production, tunable solution properties, biocompatibility, and applicability in biological and biomedical contexts [19—23]. Recently, we introduced well-defined poly(2-oxazoline)s with pendant β-D-glucose residues, referred to as glucopolyoxazolines, by controlled cationic polymerization of 2-(3-butenyl)-2-oxazoline and subsequent thiol-ene „click" glucosylation [24]. A series of glucosylated homopolymers (series A in Scheme A) [24] as well as statistical copolymers (series B) [25] and block copolymers (series C) comprising 2-isopropyl-2-oxazoline as a second hydrophilic component have been prepared. These (co)polymers were studied according to their interaction and clustering rate with concanavalin A (Con A), a plant lectin specifically binding to mannose (Man) and glucose (Glc) residues, by turbidimetric measurements. The effect of the valency (= ab-

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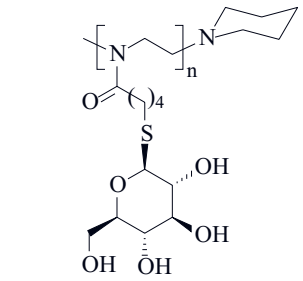
solute number of carbohydrate units, *n*) and of the carbohydrate or epitope density (= mole fraction of carbohydrate units, *x*) was systematically explored by variation of the glucopolyoxazoline chain length (series A) and composition (series A, B, and C).

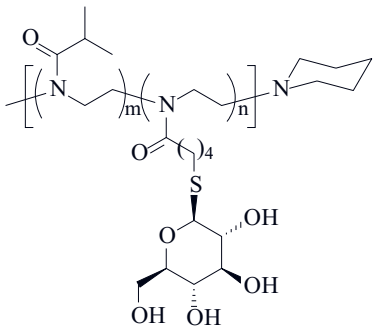
EXPERIMENTAL

Materials and polymer synthesis

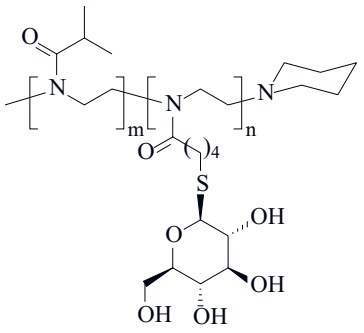
Monomers and (co)polymers were synthesized as described elsewhere [24, 25]. Exemplary procedure for **C2**: (1) To a solution of 2-isopropyl-2-oxazoline (2.30 g, 20 mmol, freshly distilled from CaH_2) in acetonitrile (18 cm^3, freshly distilled from CaH_2) under a dry argon



	A1	A2	A3
n	21	63	126
x	1	1	1
PDI	1.2	1.1	1.1



	B1	B2
m	54	69
n	6	21
x	0.10	0.23
PDI	1.1	1.1



	C1	C2
m	65	40
n	39	63
x	0.38	0.61
PDI	1.3	1.3

*Scheme A. Chemical structures of glucopolyoxazoline homopolymers **A1-3**, statistical copolymers **B1-2**, and block copolymers **C1-2**; n, m: number-average degrees of polymerization (^{1}H NMR), x = n/(m+n): mole fraction of glucose units, PDI: apparent polydispersity index (SEC)*

atmosphere were added 0.075 cm^3 (0.5 mmol) of methyl tosylate (Sigma-Aldrich). The solution was stirred at 70 °C for 2 days to reach quantitative monomer conversion. Then, 2-(3-butenyl)-2-oxazoline (30 mmol, 3.75 g, freshly distilled from CaH_2) was added *via* a syringe, and the reaction mixture was stirred for another 2 days. The polymerization was quenched by the addition of dry piperidine (threefold excess with respect to initiator). The resulting polymer was dialyzed against methanol (regenerated cellulose, MWCO: 1 kDa) and freeze-dried from a benzene/1,4-dioxane mixture (→ **C2′**). Numbers of repeating units, composition, and polydispersity index were determined by ^{1}H NMR analysis and SEC, as described earlier [24, 25]. (2) **C2′** (100 mg) and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (370 mg, Sigma-Aldrich) were dissolved in tetrahydrofuran (THF, 2.5 cm^3) under an argon atmosphere. The mixture was exposed to UV radiation (λ > 300 nm) at room temperature for 24 h. The crude sample was dialyzed against THF and freeze-dried from 1,4-dioxane (→ **C2″**). Acetyl protecting groups were removed by stirring a solution of **C2″** (50 mg) in chloroform (5 cm^3) with 0.5 cm^3 0.5 M sodium methoxide in methanol for 1 h at room temperature. The solvent was evaporated and the resulting solid dissolved in distilled water. The solution was adjusted to pH 6 with 0.1 M aqueous HCl, dialyzed against distilled water, and freeze-dried [25]. The chemical structures and molecular characteristics of the sample are given in Scheme A.

Methods of testing

Turbidimetric measurements

To 0.45 cm^3 of a polymer solution at a concentration of 1 mM per glucose unit in HEPES-buffered saline (HBS) [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 1 mM $CaCl_2$] were added 0.05 cm^3 of a freshly prepared 1 mM solution of Con A (Sigma-Aldrich) in bidistilled water (27 mg·cm^{-3}). The mixture was prepared in a UV quartz cell and the absorption at λ = 420 nm directly recorded for 30 min on a Perkin Elmer Lambda 2 UV/Vis spectral photometer.

Dynamic light scattering

DLS measurements were performed on a ALV-7004 Multiple tau digital correlator equipped with CGS-3 Compact Goniometer system, 22 mW He-Ne laser (λ = 632.8 nm) and a pair of avalanche photodiodes operated in a pseudo-cross-correlation mode. Solutions at 1 mg·cm^{-3} were prepared by direct dissolution of the polymer in either filtered (0.2 μm) bidistilled water or in filtered HBS. Solutions were further purified using online filters (1 μm) and directly measured at 25 °C at a scattering angle of 90° (20 measurements, 60 s each).

RESULTS AND DISCUSSION

Turbidimetric measurements were applied to study the kinetics of the lectin clustering mediated by gluco-

polyoxazoline ligands in HEPES-buffered saline (HBS) solution at pH 7.4. All glucopolyoxazolines dissolved in HBS as single chains (whereas they tend to form aggregates in pure water) [21, 26, 27], as confirmed by dynamic light scattering (DLS) measurements (data not shown). Following the protocol described by Kiessling *et al.* [16], a freshly prepared solution of Con A in water was mixed with glucopolyoxazoline in HBS ([Con A]/[Glc] = 1:10), and the absorbance at $\lambda = 420$ nm (A_{420}) was recorded in a UV/Vis spectral photometer over a period of 30 min. Measurements were repeated 1—2 times to check the reproducibility and reliability of the turbidity curves (results may vary depending on the quality of the lectin, mixing procedure, *etc.*).

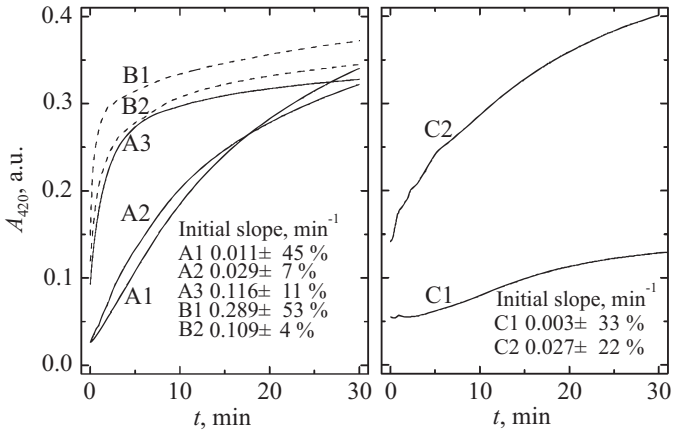


*Figure 1. Turbidimetric measurements (absorbance at λ = 420 nm versus time) for the clustering of concanavalin A with glucopolyoxazoline homopolymers **A1-3**, statistical copolymers **B1-2**, and block copolymers **C1-2**. The given initial slope values are the average values from 2-3 independent measurements*

Representative experimental turbidity curves (A_{420} versus time) for all series **A-C** are shown in Figure 1. In all cases except for **C1** (see below), the absorbance of the buffered polymer solution steeply increased upon the addition of Con A, leveling off at later times (occasionally reaching a plateau within the experimental time frame of 30 min). It is important to mention that no change in turbidity could be observed for mixtures of Con A with either poly(2-isopropyl-2-oxazoline) (zerovalent ligand) or β-D-glucose (monovalent ligand). The absorption also remained unchanged for mixtures of glucopolyoxazolines and *Ricinus communis agglutinin* (RCA I), which is a plant lectin with affinity to galactose (Gal) but not to glucose, indicating high selectivity of the ligand–protein interactions.

The results obtained for the homopolymer series **A1-3** (epitope density, $x = 1$) indicate that the clustering rate increased significantly, by about one order of magnitude (considering the initial slopes of the turbidity curves, Fig. 1), with increasing chain length and valency, $n = 21 \rightarrow 126$. Evidently, in the investigated regime, the longer is the chain of the ligand the less restricted is the coordination of a second (third, …) lectin molecule to form clusters. This trend is different to that of Kiessling *et al*. [15], who found that the clustering kinetics remained virtually unaffected by the valency of the glucosylated polynorbornenes ($n = 3 \rightarrow 143$). However, glucosylated polynorbonenes are amphiphilic homopolymers, similar to glucosylated polybutadienes [28], and might therefore be forming aggregates in an aqueous environment (not reported). Specific contributions of the length of an individual chain may be covered within the larger ensemble of chains in an aggregate, reasonably explaining these different findings.

The clustering of Con A with **A1** ($n = 21$, $x = 1$) was found to be about 10 times slower than with **B2** ($n = 21$, $x = 0.23$), exhibiting the same valency but different epitope density. Considering the studies of Kiessling *et al*. [16] and Haddleton *et al*. [17], one would have expected the inverse trend with the precipitating of Con A occurring faster with **A1** than with **B2**. However, the two ligands are not only different in epitope density but also in chain length (and chemical composition, see below). A fully stretched **A1** chain just spans ~7.4 nm (**B2**: ~32 nm), which is less than the hydrodynamic diameter of Con A (8.6 nm, DLS) [29]. The observed differences in clustering kinetics could therefore be simply due to a steric restriction to form larger clusters (see above). On the other hand, the precipitation rates in the series **B1-2** of statistical copolymers were found to increase with decreasing epitope density, $x = 0.23$ (**B2**) $\rightarrow 0.10$ (**B1**; contour length ~21 nm). Evidently, clustering occurs faster the larger is the spacing between the glucose units. The average distances between glucose residues are about 3.5 nm (**B1**) and 1.5 nm (**B2**), assuming an even distribution of glucosylated units along the polymer chain [25] and a monomer segment length (all-*trans* -N–C–C-) of 0.35 nm, which is still smaller than the distance of ~6.5-7.2 nm between the binding sites of Con A. [30, 31].

The reason for the opposite behaviors in clustering kinetics observed in the different studies is not known yet. It is hypothesized that this could be due to different states of aggregation (see above) and/or chemical compositions of the ligands. Notably, recent studies by Hartmann *et al*. [31] suggest that the binding of sequence-defined glyco-oligomers to Con A does not only depend on the number and spacing of presented carbohydrates but also on the chemical composition of the polymer backbone. **B1-2** consist of a polyamide backbone with pendant glucose binding units and isopropyl non-binding units (see Scheme A), whereas the glycosylated polynorbornenes [16] and polymethacrylates [17] carry mannose binding units and galactose non-binding units, *i.e.* carbohydrates at every repeating unit. Especially the different non-binding units might have a particular effect on clustering

kinetics, whether or not they contribute to a shielding of the binding units.

Finally, two block copolymers **C1** ($n = 39$, $x = 0.38$) and **C2** ($n = 63$, $x = 0.61$) were examined. Clustering rates with **C2** and the homopolymer **A2** ($n = 63$) are rather similar, suggesting that the non-binding block could have a minor effect on clustering kinetics. However, the clustering of Con A with **C1** was found to be extremely slow, even slower than with **A1** ($n = 21$). Supposedly, for $m > n$, the non-binding block can efficiently shield the glucose residues and prevent complexation with Con A.

In summary, the clustering kinetics of Con A using glucosylated polyoxazolines (Scheme A) was found to be affected by the structure of the ligand. For homopolymers, and also block copolymers, a considerable impact of the chain length and valency on the lectin interaction was noticed. For statistical copolymers, the clustering was faster for lower epitope densities or wider spacings between glucose residues. It further appeared that also the nature of the non-binding unit had impact on the clustering kinetics.

Turbidimetry is a common probe to evaluate lectin–ligand interactions but has limitations in the quantitative measurement of kinetic parameters. Kinetic studies shall be extended by surface plasmon resonance (SBR) and complemented by determination of thermodynamic parameters using isothermal titration calorimetry (ITC) [32].

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