Influence of freezing on physical stability of Rituximab

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Introduction

Rituximab (RTX) is a chimeric monoclonal antibody (mAb) widely used in the treatment of various haematological malignancies. Our team has recently demonstrated that RTX solutions are physically, chemically and microbiologically stable at + 4 C for 6 months (1). Freezing, even attractive, is generally not considered as an appropriate way to conserve proteins due to possible denaturation. Since few data are available on the influence of freezing on the physical stability of mAbs, the goal of our study was to understand the influence of freezing on RTX stability.

Tools and Methodology

2 ml aliquots of 10 mg/ml solution of RTX in PP tubes were submitted to one cycle of freezing/thawing. Two methods have been used: static freezing (SF) at -22 C and liquid nitrogen cryonics (LNC), both followed by a room temperature thawing. Physical stability has been evaluated by UV spectrophotometry, Dynamic Light Scattering (DLS) , Size exclusion Chromatography (SEC) and FTIR spectroscopy (FTIR). Results obtained by different ways of freezing were compared to those who were not frozen.

Results

UV spectrophotometry

	OD at 279 nm	
RTX not frozen	1.0173	0.0031
SF	0.9807	0.0006
LNC	0.9811	0.0002

For both freezing modes, absorbance decreased at 279 nm as compared to reference. This weak decrease confirms the visual aggregation.

Turbidimetry

	OD at 350 nm	
RTX not frozen	0.0873	0.0002
SF	0.1014	0.0002
LNC	0.0956	0.0001

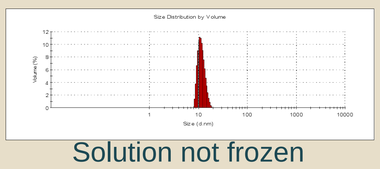
For static freezing and liquid nitrogen DO at 350nm increased (0.0873 0.0002 (reference) vs. 0.1014 0.0002 (SF) and 0.0956 0.0001 (LNC) in accordance to aggregation.

Dynamic light scattering (hydrodynamic diameter)

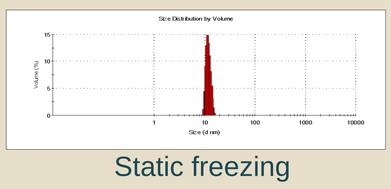
		Pic 1		
		Median (nm)	Polydispersity index	
T0	RTX not frozen	12.62 0.12	0.058	Monodisperse
1 cycle	SF	12.35 0.14	0.164	Polydisperse
	LNC	12.09 0.56	0.177	Polydisperse

Only one peak corresponding mainly to rituximab monomer is observed
Only one polydisperse population (polydispersity index > 0.1 at diameter 12.35 0.27 nm) was observed by DLS after freezing, suggesting that visual aggregates had a size above the maximum detection range (6µm)

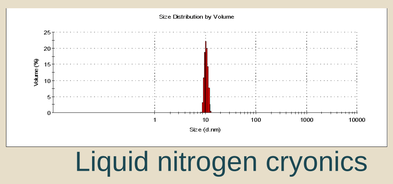
Distribution in size of diameters obtained for:



Solution not frozen



Static freezing

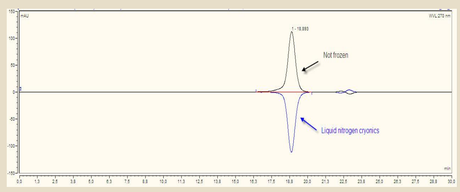


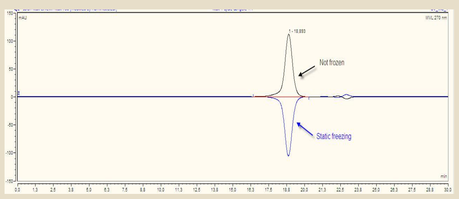
Liquid nitrogen cryonics

Size exclusion Chromatography

	Retention time t_R (min)	AUC* (mAU.min)
RTX not frozen	18.921 0.021	76.8648 2.699
SF	18.910 0.079	69.8292 1.297
LNC	18.877 0.004	70.5178 0.768

No retention time shift or additional peak were observed by SEC after centrifugation, but a significant decrease in AUC was observed (76.864 2.699 vs. 69.82 1.297 (SF) and 70.51 0.768 (LNC), confirming a loss by micronic aggregation (about 9.1 %).



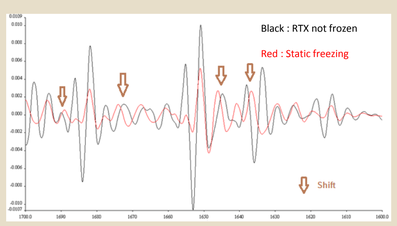


Mirror chromatogramm obtained for samples:
- Static freezing (on the left)
- Liquid nitrogen cryonics (on the top)

FTIR spectroscopy

	RTX not frozen	SF	LNC
β -Sheet(%)	59.35 ± 0.23	59.03 ± 1.69	54.19 ± 0.16
β-turn(%)	29.86 ± 0.19	28.28 ± 1,67	30.25 ± 0.06
Random (%)	10.83 ± 0.10	10.09 ± 0.31	10.30 ± 0.13
α helix (%)	0	3.44 ± 0.17	5.26 ± 0,02

For both freezing modes, IR showed numerous shifts revealing 2 destabilization ways of II structure of rituximab, with the appearance of α-helix



Conclusion

For both SF and LNC, it has been demonstrated that a freezing/thawing cycle alters RTX in solution (aggregation and secondary structure modifications), so freezing is not a proper method for rituximab conservation. Finally, our study point out that accidental freezing of RTX is probably harmful and should be avoided.

Reference

1. Paul et al, Long-term stability of diluted solutions of the monoclonal antibody rituximab. Int J Pharm ; 436, 1-2: 282-290. 2012

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