EVALUATION OF AFFINITY MEMBRANE ADSORBERS FOR ANTIBODY PURIFICATION

Cristiana Boi[1], Simone Dimartino[1], Peter Van Beijeren[2] and Giulio C. Sarti[1]
(1) Chemical Engineering, Mining and Environmental Technology, University of Bologna, viale Risorgimento 2, Bologna, Italy,
(2) Biochemical and Chemical Engineering, University of Dortmund, Dortmund, Germany

Introduction

Membrane chromatography is a novel protein purification technique developed to overcome the major limitations due to packed beads, such as long process time, mass transport controlled by diffusion and high pressure drops.

In this work, the adsorption of human IgG onto A2P Sartoepoxy membranes has been studied. To this aim, several breakthrough curves, including elution, have been measured at different experimental conditions. Furthermore, subsequent cycles have been performed to evaluate the stability of A2P Sartoepoxy membranes.

In order to investigate the influence of pH on the elution peaks, two different elution buffers have been used. Moreover, elution was also performed in batch to evaluate the amount of protein loaded onto the different membranes as function of their position in the stack.

Experimental

Materials and method

Pre-activated cellulose membranes, Sartobind epoxy (Sartorius AG, Göettingen, Germany), have been used as supports for the immobilization of aminoalkyl linked A2P mimetic ligand (Prometic Biosciences, Cambridge, UK); coupling was performed by Prometic Biosciences. The membranes were cut into circles of 2.5 cm diameter and washed twice with PBS before use in dynamic experiments. Human IgG was purchased from Octapharma (Sweden); IgG samples were prepared by diluting the concentrated solution in PBS buffer. The protein concentration of each sample was determined by UV absorption at 280 nm by using a UV-Visible Spectrophotometer, Shimadzu UV-1601. Dynamic experiments have been performed using an FPLC AKTA Purifier 100 (GE Healthcare, UK). A solution of 0.1 M glycine pH 2.8 and a solution of 0.1 M citric acid pH 2.5 were used as elution buffers.

Dynamic breakthrough curves

Several dynamic experiments have been performed to evaluate the influence of feed protein concentration, flow rate, subsequent cycles and membrane regeneration on the breakthrough curves (BTC). All such experiments have been performed with a stack of ten membranes and using the glycine solution as elution buffer. The membranes have been regenerated in batch with 1 M NaOH for 45 minutes, after the 2nd or after the 3rd cycle. Table 1 summarizes the experimental conditions of the dynamic experiments, whilst Figures 1-3 show the loading, washing and elution curves at a flow rate of 1 ml/min and at feed concentration of 0.5 mg/ml.

The dynamic binding capacities (DBC) at 100% breakthrough, the protein amount eluted and the recovery are summarized in tables 2-5. The recovery is defined as the ratio between the DBC based on elution and the DBC based on adsorption.

Table 1. Experimental conditions of dynamic experiments.

Flow rate [ml/min]	IgG concentration [mg/ml]	Total cycles measured	Regeneration after cycle
0.50	0.50	5	2
0.50	0.25	5	3
1.0	0.50	6	3
1.0	0.25	6	3

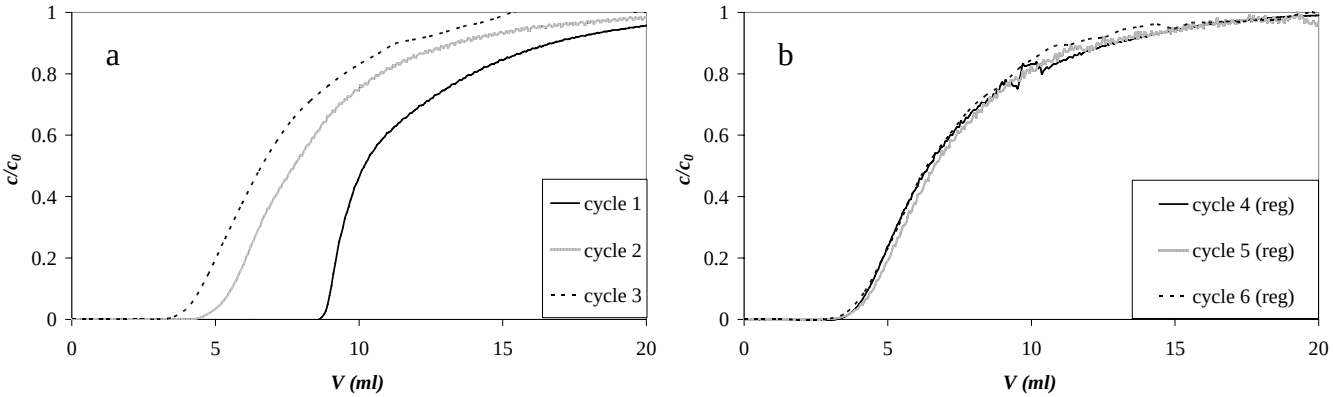

Figure 1. Loading step for six subsequent breakthrough curves of IgG on A2P Sartoepoxy membranes at 1 ml/min and 0.5 mg/ml: (a) before regeneration; (b) after regeneration.

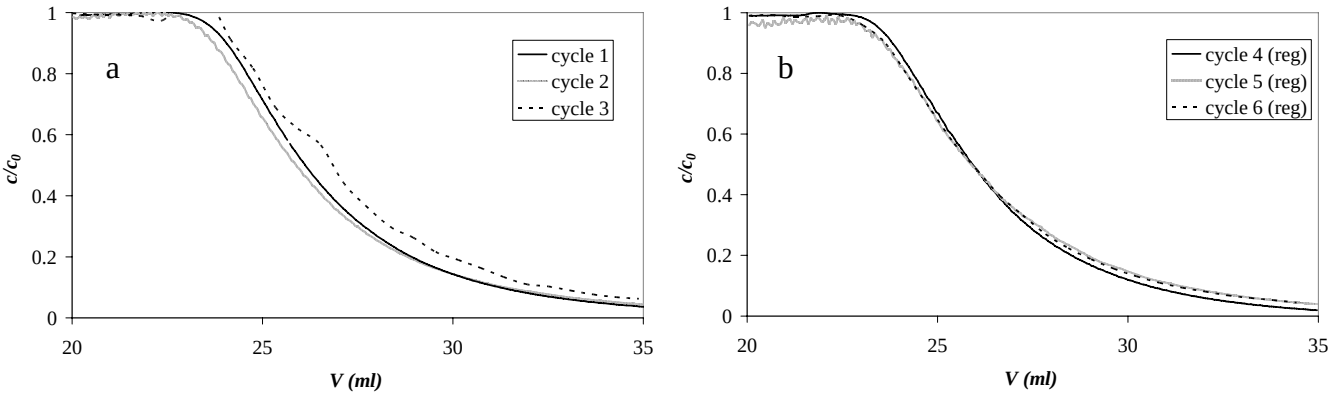

Figure 2. Washing step for six subsequent breakthrough curves of IgG on A2P Sartoepoxy membranes at 1 ml/min and 0.5 mg/ml: (a) before regeneration; (b) after regeneration.

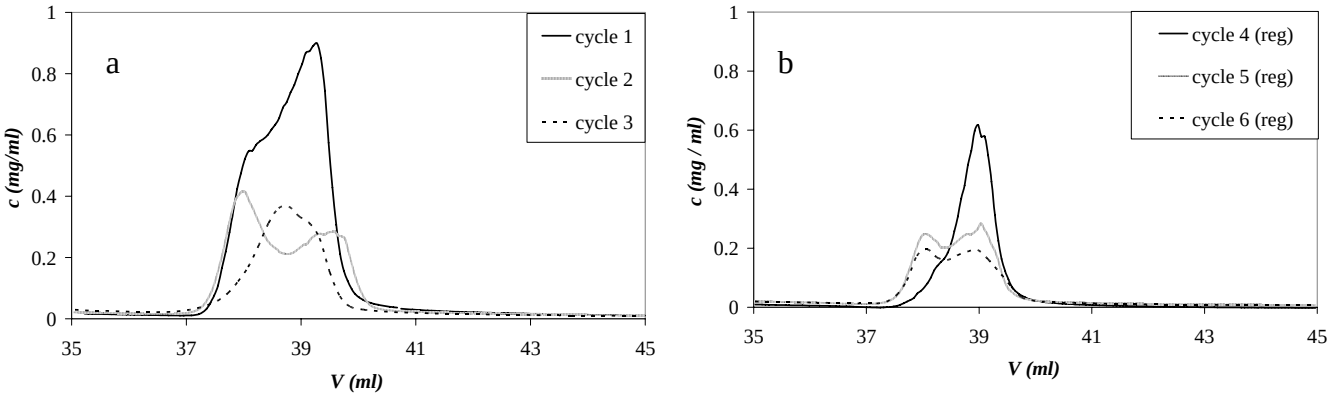

Figure 3. Elution step for six subsequent breakthrough curves of IgG on A2P Sartoepoxy membranes at 1 ml/min and 0.5 mg/ml: (a) before regeneration; (b) after regeneration.

Table 2. Dynamic binding capacities and recovery at 0.5 ml/min and 0.5 mg/ml.

	1	2	3	4	5
DBC Ads [µg/cm^2]	43.3	16.8	12.1	14.2	4.0
DBC Elu [µg/cm^2]	27.4	11.1	12.2	9.4	8.6
Recovery [%]	63.3	66.1	100.3	65.9	-

Table 3. Dynamic binding capacities and recovery at 0.5 ml/min and 0.25 mg/ml.

	1	2	3	4	5
DBC Ads [µg/cm^2]	40.7	8.7	8.7	8.5	8.0
DBC Elu [µg/cm^2]	24.3	7.2	5.4	7.4	8.5
Recovery [%]	59.6	83.5	61.5	87.0	106.0

Table 4. Dynamic binding capacities and recovery at 1 ml/min and 0.5 mg/ml.

	1	2	3	4	5	6
DBC Ads [µg/cm^2]	48.8	18.9	13.8	10.1	10.3	8.0
DBC Elu [µg/cm^2]	29.5	16.8	11.8	10.3	9.6	8.0
Recovery [%]	60.5	88.9	85.6	102.2	93.1	100.1

Table 5. Dynamic binding capacities and recovery at 1 ml/min and 0.25 mg/ml.

	1	2	3	4	5	6
DBC Ads [µg/cm^2]	49.8	17.6	11.2	15.1	7.0	7.2
DBC Elu [µg/cm^2]	27.3	15.5	10.7	16.4	14.0	11.4
Recovery [%]	54.8	87.7	95.7	108.5	-	-

Figures 1-3 and Tables 2-5 clearly show that during the first cycle breakthrough occurs much later than in subsequent cycles, indicating that the dynamic binding capacity decreases after use. Moreover, in the first cycles only 60% of the adsorbed protein is recovered with elution.

After regeneration the capacity is not regained as expected. Dynamic binding capacities for the first cycles after regeneration ranged between 8.5 and 15.1 μg/cm^2, and recovery was significantly higher compared to the first cycles of the new membranes.

The effects of different flow rates and concentrations on the response of new membranes are shown in Figures 4-5, respectively.

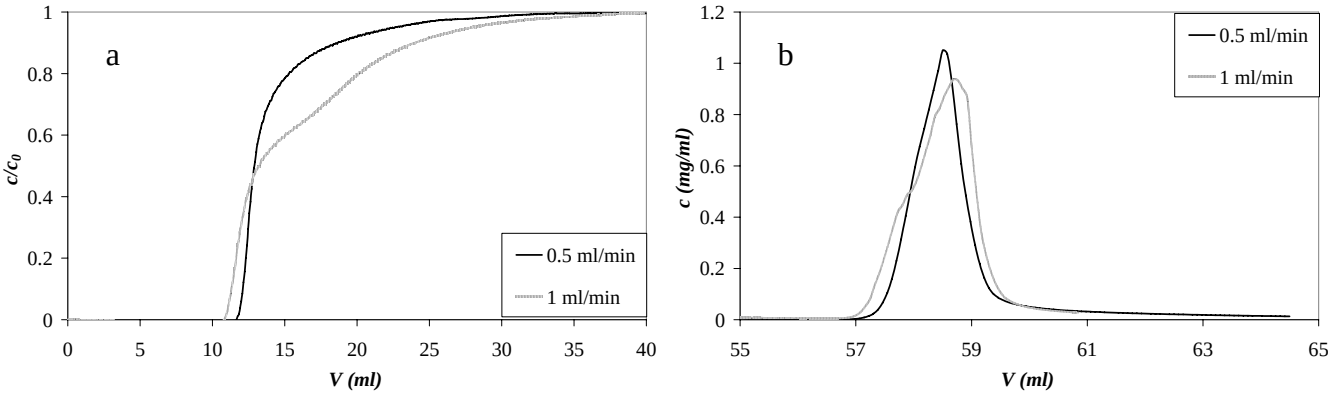

Figure 4. Comparison of the 1st cycle at 0.25 mg/ml and different flow rates: (a) adsorption step; (b) elution step.

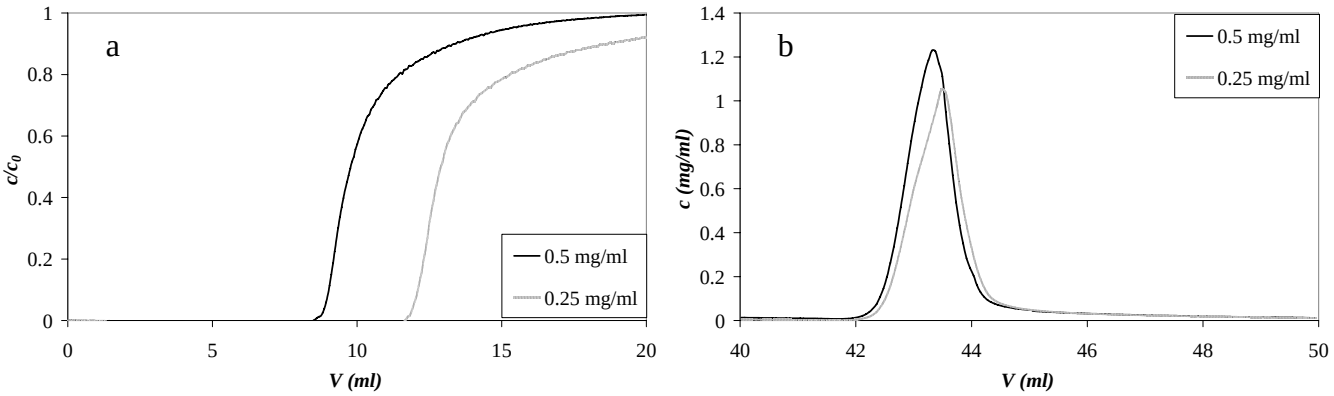

Figure 5. Comparison of the 1st cycle at 0.5 ml/min and different feed concentration: (a) adsorption step; (b) elution step.

Apparently, the initial breakthrough volume is unaffected by increasing the flow rate from 0.5 ml/min to 1 ml/min, while the DBC shape is modified in the higher volume range. As expected, the elution peaks become shorter and broader with increasing flow rate. The amount eluted is independent of flow rate, as it is clear from Tables 2-5. At the higher flow rate the DBC is larger; this appears an interesting result, for which, however, the physical motivation is to be found.

As it is shown in Figure 5a, at higher feed concentration the BTC is delayed at higher volumes, while the elution peaks are almost the same.

Influence of elution buffer

With the aim to improve recovery, BTC have been measured at the concentration of 0.25 mg/ml, at the two flow rates of 0.5 ml/min and 1 ml/min, using citric acid as the elution buffer. The results are compared with the experiments performed using glycine. Two subsequent cycles have been considered, with no intermediate regeneration.

The elution curves are showed in Figure 6, while the DBC and the recoveries for the two elution buffers are summarized in Tables 6 and 7.

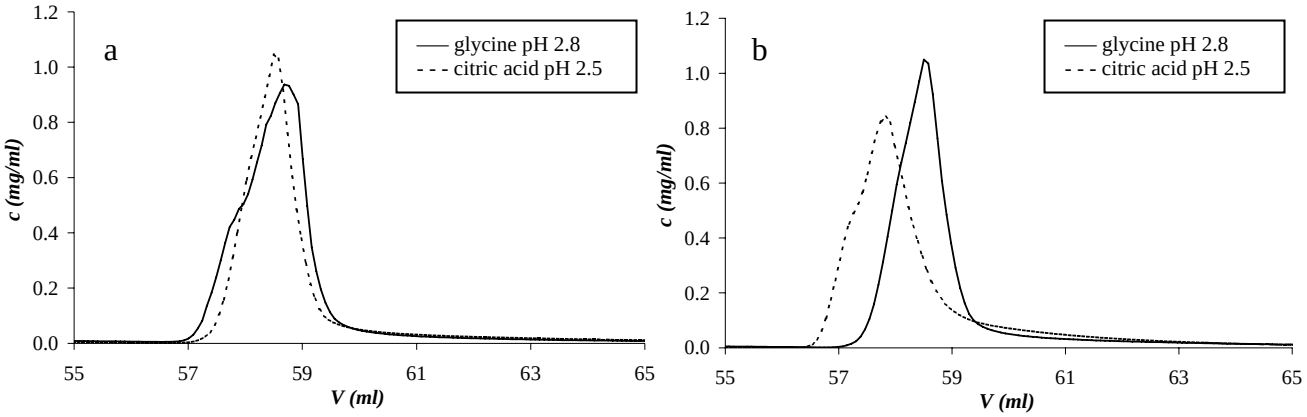

Figure 6. Comparison of different elution buffers. Elution step of 1st cycle at 1 ml/min (a), 0.5 ml/min (b) and feed concentration of 0.25 mg/ml.

Table 6. Comparison of elution buffers at 0.25 mg/ml and 0.5 ml/min.

	Cycle_1 Glycine	Cycle_1 Citric acid	Cycle_2 Glycine	Cycle_2 Citric acid
DBC Ads [μg/cm^2]	40.7	45.3	8.7	10.0
DBC Elu [μg/cm^2]	24.3	25.8	7.2	10.1
Recovery [%]	59.6	56.8	83.5	101.0

Table 7. Comparison of elution buffers at 0.25 mg/ml and 1 ml/min.

	Cycle_1 Glycine	Cycle_1 Citric acid	Cycle_2 Glycine	Cycle_2 Citric acid
DBC Ads [μg/cm^2]	49.8	41.6	17.6	16.1
DBC Elu [μg/cm^2]	27.3	24.5	15.5	14.5
Recovery [%]	54.8	58.9	87.7	89.8

The elution curves obtained with the two buffers are rather similar, and the albeit small pH differences did not produce noticeable improvements. Capacity loss over multiple cycles is also similar for both elution buffers.

Batch Elution Experiments

To investigate the amount of IgG bound onto the membranes as a function of their position in the stack, the elution procedure was changed from continuous to batch mode. After the washing step, the membranes were taken out of the membrane holder and elution was performed batch wise. All experiments have been performed with a stack of 10 membranes, which were split up in five pairs of two membranes each, during batch elution. The elution buffer is 0.1 M glycine with pH 2.8. The IgG feed concentration was approximately 0.5 mg/ml and the flow rate was 1 ml/min for the adsorption and washing steps performed in dynamic conditions.

Two experiments have been performed. The first experiment (labelled A) consists of two cycles: during the first cycle had a continuous elution step, while in the second cycle elution was performed in batch mode. No regeneration of the membranes has been performed. In the second experiment (labelled B), the membranes were regenerated between the two cycles. New membranes have been used for both experiments. The experimental results thus obtained are shown in Figure 7 and Table 8.

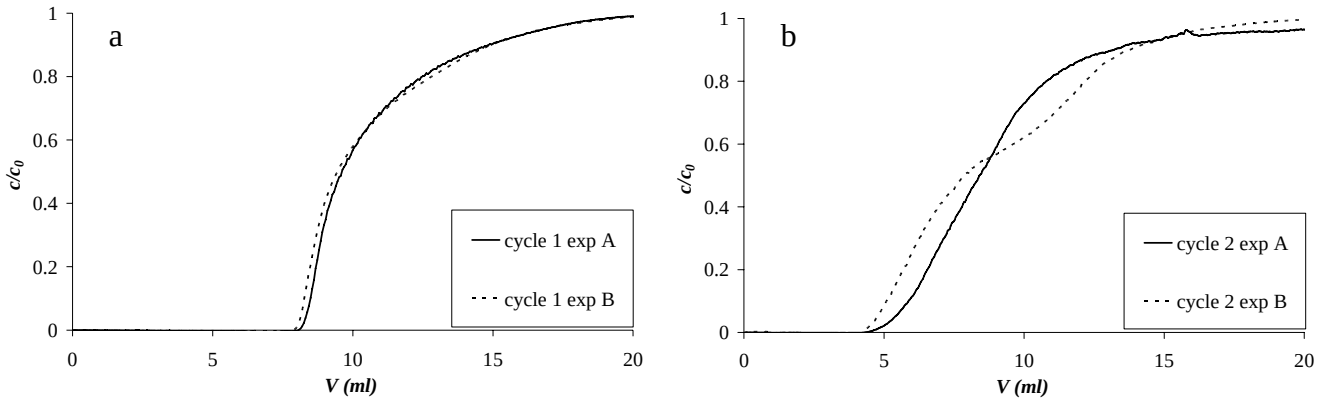

Figure 7. Comparison of two subsequent breakthrough curves for experiment A and experiment B: (a) 1st cycle; (b) 2nd cycle.

Table 8. Dynamic binding capacities and recoveries for experiment A and B.

(a) 1st cycle	exp A	exp B
DBC Ads [µg/cm^2]	44.7	46.8
DBC Elu [µg/cm^2]	33.3	33.7
Recovery [%]	74.4	71.9

(b) 2nd cycle	exp A	exp B
DBC Ads [µg/cm^2]	25.1	30.4
DBC Elu [µg/cm^2]	27.3	26.8
Recovery [%]	108.8	88.2

Regeneration affects the amount adsorbed in the second cycle; however, the amounts eluted in the second cycle of both experiment A and B are very similar. This is consistent with the fact the ligand used can bind both through affinity sites and also through non specific bonds; the former are subject to elution with the buffer used, while the non specific bonds are broken only during the regeneration step and not during elution. As a consequence, the amount adsorbed after regeneration is higher, but the amount eluted remains the same both for regenerated and non-regenerated membranes.

The elution results from membranes in different positions in the stack are presented in Table 9.

Table 9. DBCs based on batch elution as function of axial location, #1, 2 represents the first two membranes close to the inlet and #9, 10 labels the membranes close to the outlet.

(a) 1st cycle	exp A	exp B
DBC Elu #1, 2 [μg/cm^2]	-	38.4
DBC Elu #3, 4 [μg/cm^2]	-	33.0
DBC Elu #5, 6 [μg/cm^2]	-	30.6
DBC Elu #7, 8 [μg/cm^2]	-	32.1
DBC Elu #9, 10 [μg/cm^2]	-	34.4

(b) 2nd cycle	exp A	exp B
DBC Elu #1, 2 [μg/cm^2]	35.7	32.6
DBC Elu #3, 4 [μg/cm^2]	26.1	25.5
DBC Elu #5, 6 [μg/cm^2]	24.9	25.0
DBC Elu #7, 8 [μg/cm^2]	24.6	25.8
DBC Elu #9, 10 [μg/cm^2]	25.2	25.1

Table 9 shows that the amount of protein eluted is larger for the first membranes in the stack, closer to the feed inlet, while for all the other membranes there is no effect of position, both for regenerated and non regenerated membranes. That behavior seems associated more to flow distribution non uniformities than to intrinsic properties of the membranes, and is currently under investigation.

Conclusions

The subsequent separation cycles, performed with the A2P-Sartoepoxy affinity membranes examined, indicate that after the first cycle the DBC is significantly reduced to about one third of the value measured for the new membranes. In parallel, the protein recovery after elution is about 60 % of the adsorbed protein, for the first cycle and increases in subsequent cycles.

The behavior observed suggests the existence of specific activity binding as well as non-specific binding of IgG onto the A2P affinity membranes used. The higher capacity measured in the first cycle, apparently results from both specific and non-specific binding, such as hydrophobic binding. The elution step applied would recover only the protein specifically bound, leaving onto the membrane the amount adsorbed through non-specific binding. A subsequent cycle will thus find a surface with lower capacity for non-specific protein

adsorption, but virtually similar availability for specific sites for IgG; therefore the overall capacity is decreased with respect to the first cycle and, at the same time, the recovery during elution is increased.

That is also supported by the behavior observed after membrane regeneration. Indeed, regeneration will detach also the protein adsorbed through non-specific bonds over the surface, thus increasing the overall capacity to values close to those of the new membranes, since both specific and non-specific bonds are restored after regeneration.

Interestingly, by increasing the number of subsequent cycles the elution peak consists of double peaks, or at least have the shape of two overlapping elution peaks, reasonably indicating the coexistence of two different elution kinetics. The latter may be associated to two different binding mechanisms.

Ligand leakage could also play a role in understanding the capacity loss in subsequent cycles, but it was not possible to detect reliably this possibility for the lack of suitable specific analytical methods. This aspect is still under investigation.

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