MONITORING ACTION READINESS OF COUPLING REAGENT CARBODIIMIDE IN AQUEOUS SOLUTIONS

[1,2]Daniel ŽATKO, [2,3]Jakub VĚŽNÍK, [2]Karel LACINA

[1]*Masaryk University, Department of Biochemistry, Faculty of Science, Brno, Czech Republic, EU,* *danielzatko99@gmail.com*

[2]*Masaryk University, Central European Institute of Technology, Brno, Czech Republic, EU*

[3]*Masaryk University, Department of Chemistry, Faculty of Science, Brno, Czech Republic, EU*

https://doi.org/10.37904/nanocon.2023.4806

Abstract

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is the most used carbodiimide for conjugating biological substances containing carboxyls and amines. The use of EDC for conjugating a protein containing amines to a particle with a carboxyl group is often utilized for the preparation of modified nanoparticles, which are used for example in electrochemical immunosensing[1]. EDC is an unstable substance that undergoes hydrolysis in aqueous environments, which can lead to reduced formation of the desired conjugate. The most significant complication in the complex reaction occurs during the generation of the active intermediate O-acylisourea, which is formed in seconds in the solution. Subsequent hydrolysis and the consequent reformation of the carboxyl group prevent the reaction from progressing, resulting in no conjugate formation. Due to this limitation, there is motivation to monitor the stability of EDC in aqueous environments and to develop a simple method for rapidly determining the activity and functionality of stored EDC. We have developed a new simple precipitation method for semi-quantitative determination of the activity of EDC in aqueous solutions and tested it against the established pyridine method. The potential of our approach for rapidly assessing the active state and functionality of the EDC lies in the simplicity of its preparation and instrument independent usage.

Keywords: Bioconjugation, O-acylisourea, protein bioconjugation, 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide hydrochloride

1. INTRODUCTION

For decades, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) has been one of the most popular compounds for conjugating biological substances containing carboxylates and amines. EDC-mediated reactions have propelled advancements in fields such as immunology, proteomics, drug development, and biosensing [2]–[4].

However, EDC carbodiimide undergoes hydrolysis in an aqueous solution. The main practical problem of the hydrolysis reaction is the formation of undesired side products, leading to decreased reaction efficiency, and, in some cases, a loss of the intended bioconjugation target. Only a few studies have shown procedures to measure carbodiimides hydrolysis [5]–[7]. A method for monitoring EDC carbodiimide can be a useful tool for bioconjugation chemistry.

2. METHODOLOGY

A semi-quantitative method was developed to monitor the hydrolysis of carbodiimide EDC in aqueous media, which can be implemented without the use of sophisticated measuring devices. Measurements of the

decreasing absorbance during the clearance of the EDC solution were used to study and validate this method. The pyridine method was used for confirmation [8]. The measurement of absorbance indicates the degree of hydrolysis of EDC. This procedure can also be used for semi-quantitative determination of activity based on visual observation of turbidity when EDC is mixed with a polycarboxyl entities (polyacrylic acid, carboxylated nanoparticles).

3. EXPERIMENTAL

3.1 Precipitation method

The solution employed for the precipitation method contained 50 mM EDC (ThermoFisher) in 100 mM MES buffer, pipetted into a 96-well plate for experimental measurement of absorbance in a plate reader (Uniogen, Finland). 50 µl of the solution of EDC was mixed with 50 µl of 5 wt% solution of polyacrylic acid (PAA) (Sigma Aldrich) in 100 mM MES buffer directly in the well of the microplate. Alternatively, additional variation of the precipitation method was studied with the addition of 50 mM isobutylamine (ThermoFisher). Another variation of the precipitation method was the use of 0.5 wt% carboxylated polystyrene carbon nanoparticles (Magsphere Inc., U.S.A.) instead of polyacrylic acid.

3.2 Pyridine method

Standard assay conditions for the colorimetric assay procedure to detect EDC activity was 10 µl of EDC solution pipetted into a 96-well plate for absorbance measurement in a plate reader. The colour-developing mixture contained 100 µl of 2 M pyridine (Sigma Aldrich), HCl, and 1 M 1,2-diaminoethane (Sigma Aldrich) at pH 7.0. The absorbance was measured at 400 nm, the wavelength at which there is the highest absorption [8].

4. RESULTS AND DISCUSSION

Initially, we hypothesised that the precipitation occurs by crosslinking the carbodiimide activated carboxyl groups of PAA or nanoparticles by ethylenediamine. However, a control experiment showed that ethylenediamine forms precipitate with polyacrylic acid even in the absence of EDC (figure not shown). Another approach to precipitation tested conjugation of isobutylamine to activated carboxylic groups with the intent to reduce the polarity of PAA. The reaction was carried out by mixing 50 µl of isobutylamine, EDC, and EDC/sulfo-NHS. All compounds were mixed to give 50 mM concentration, with 50 µl of 5 % PAA in 100 mM MES (**Figure 1**). We devised a simple test to determine practical useability of this assay, solution of EDC was “forgotten” in the laboratory (50 mM EDC in MES, 24 h at lab temperature) and no precipitation occurred in our assay with this solution of EDC (**Figure 1**, green curve).

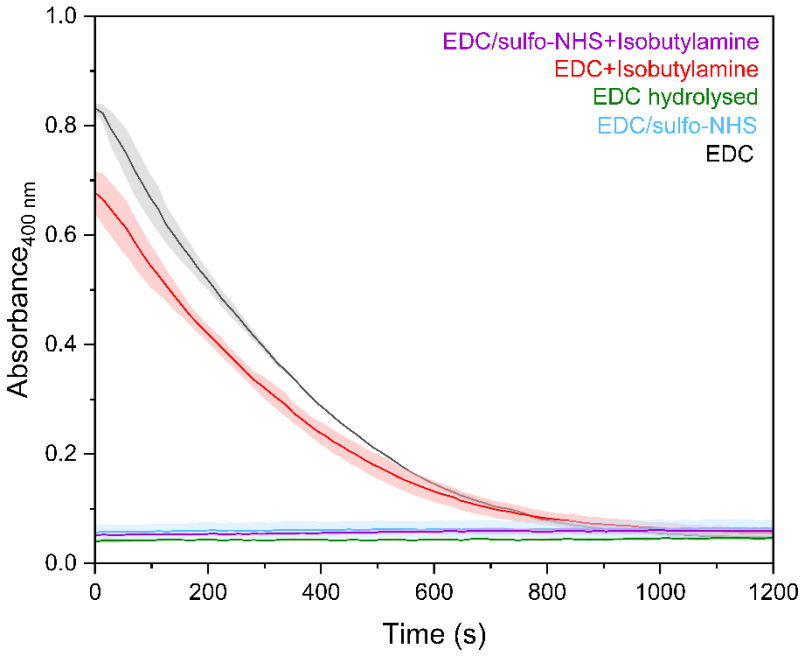

Figure 1 Time course of absorbance at 400 nm for EDC precipitation method using diverse combinations of specific reagents. Isobutylamine shows minimal, nearly negligible impact on precipitation.

Surprisingly, immediate precipitation also occurred when just EDC was mixed with PAA. The precipitate that is formed is unstable and completely dissolves in 15 minutes. Based on this we suggest that the precipitation occurs due to interaction of negatively charged carboxylic groups of PAA and positively charged *O*-acylisourea intermediate that is formed after activation of PAA with EDC. Similar results were observed with carboxylated nanoparticles (not shown) and in mixture of EDC + isobutylamine added to PAA. Interestingly, no precipitate is formed, when sulfo-NHS is added to the reaction mixture, since the formed sulfo-NHS ester is negatively charged.

Another goal we had in mind was the simplification of manual procedures in activation of carboxyl groups, thus aliquots of EDC in MES were prepared, and frozen. The aim of this study was also to confirm and determine whether the frozen aliquots of EDC remain active and for how long time these can be stored. To ascertain this activity, the measurement of the pre-prepared solution was divided into four weeks during which the prepared aliquots and 50 mM EDC were stored in the freezer to prevent hydrolysis. It can be seen in **Figure 2A** that the activity of carbodiimide EDC does not decrease dramatically when stored in the freezer even after four weeks, and hence hydrolysis of EDC is minimal.

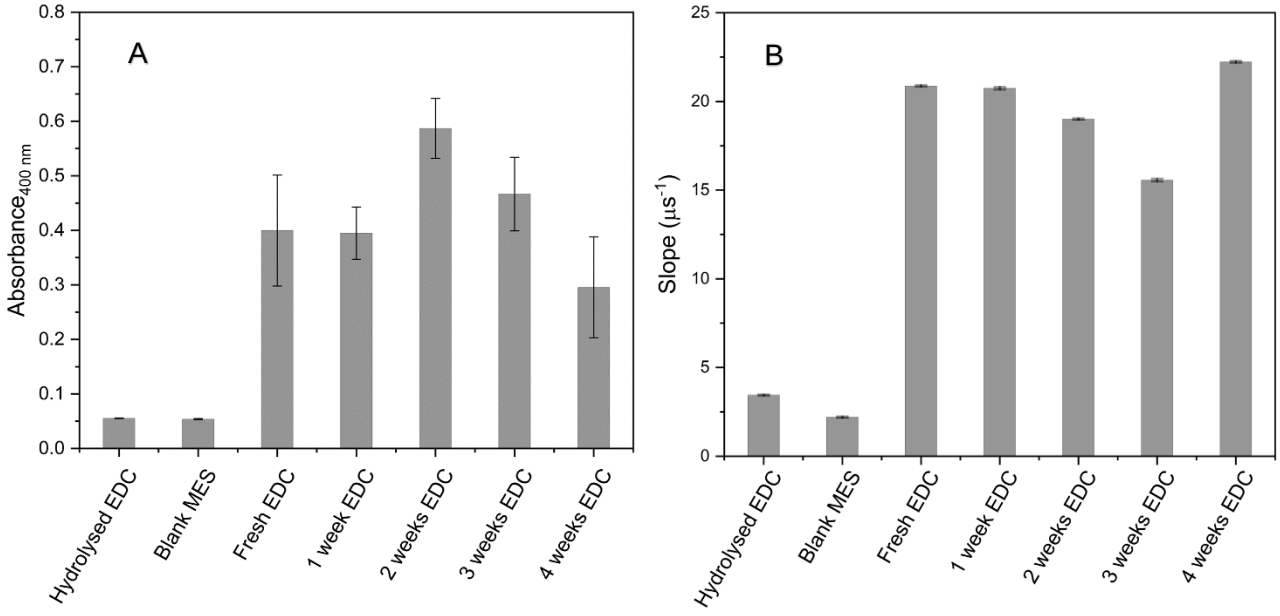

Figure 2 A: Absorbance dependence at 400 nm for long-term measurements of EDC hydrolysis by precipitation method (reading 8 minutes after mixing). **B:** Slope dependence for long-term measurements of EDC hydrolysis by pyridine method (reading after 8 minutes of measurement). Small loss of activity of frozen aqueous solution of EDC is observed for both methods.

To verify the results of the developed precipitation method, a comparative colorimetric procedure testing the activity of EDC carbodiimide was employed. This method is based on the formation of a chromophore during the colorimetric reaction of a pyridine mixture with EDC [8]. A parallel experiment with the pyridine method also showed that after four weeks of storage in the freezer no significant reduction in activity was found for aqueous EDC solution (**Figure 2B**).

The comparative pyridine method is based on the continual formation of non-specified chromophore due to which absorbance at 400 nm is elevated. The experimental data for the calculation of the slope (which is visualized in **Figure 2B** and compared with **Figure 2A)** is visible in **Figure 3**. The assessment of EDC activity based on storage conditions was conducted by subjecting EDC samples to laboratory temperature, refrigeration, and freezing (**Figure 3**). The graph depicts two-hour measurement of absorbance for EDC solutions stored at different conditions over the course of one day. In each experiment, a blank sample of MES

buffer solution was added, which did not induce any chromophoric reaction, resulting in an almost negligible change in absorbance over time.

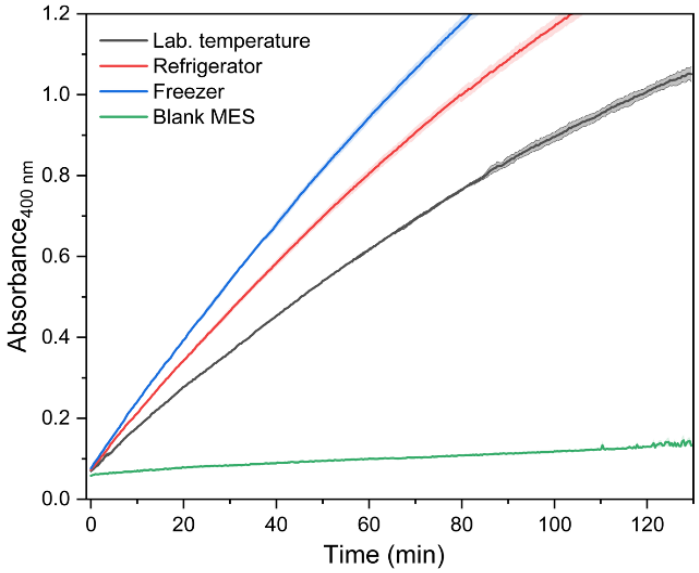

Figure 3 Absorbance dependence at 400 nm on time for different methods of storage, pyridine method. EDC left for one day at laboratory temperature, at 4°C in refrigerator and at -20°C in freezer. The most significant hydrolysis, leading to a loss of activity, is evident during the storage of EDC at laboratory temperature.

5. CONCLUSION

The precipitation method can be used as a semi-quantitative method for the determination of EDC activity in aqueous solutions. Negatively charged polycarboxylic acid forms precipitate with itself when a part of its negatively charged groups is replaced with positively charged O-acylisourea ester, which is formed by active EDC in solution. The turbidity of precipitated solution can be measured by instrument or even observed by naked eye for a rapid determination of solution activity. If hydrolysed EDC is used in the reaction, the solution stays clear. The performance of the developed method was proven in comparison with established colorimetric pyridine method.

REFERENCES

[1] SOPOUŠEK, J.; VĚŽNÍK, J.; SKLÁDAL, P. and LACINA, K. 'Blocking the Nanopores in a Layer of Nonconductive Nanoparticles: Dominant Effects Therein and Challenges for Electrochemical Impedimetric Biosensing'. *ACS Appl. Mater. Interfaces*. Mar. 2020, vol. 12, no. 12, pp. 14620–14628, Available from: https://doi.org/10.1021/acsami.0c02650.

[2] OLSSON, N.; JAMES, P.; BORREBAECK, C. A. K. and WINGREN, C. 'Quantitative Proteomics Targeting Classes of Motif-containing Peptides Using Immunoaffinity-based Mass Spectrometry'. *Mol. Cell. Proteomics*. Aug. 2012, vol. 11, no. 8, pp. 342–354. Available from: https://doi.org/10.1074/mcp.M111.016238.

[3] JAETAO, J. E. *et al.* 'Enhanced Leukemia Cell Detection Using a Novel Magnetic Needle and Nanoparticles'. *Cancer Res.* Nov. 2009, vol. 69, no. 21, pp. 8310–8316. Available from: https://doi.org/10.1158/0008-5472.CAN-09-1083.

[4] TYMIANSKI, M. *et al.* 'A novel use for a carbodiimide compound for the fixation of fluorescent and non-fluorescent calcium indicators in situ following physiological experiments'. *Cell Calcium*. Mar. 1997, vol. 21, no. 3, pp. 175–183. Available from: https://doi.org/10.1016/S0143-4160(97)90042-7.

[5] WROBEL, N.; SCHINKINGER, M.; and MIRSKY, V. M. 'A Novel Ultraviolet Assay for Testing Side Reactions of Carbodiimides'. *Anal. Biochem.* Jun. 2002, vol. 305, no. 2, pp. 135–138. Available from: https://doi.org/10.1006/abio.2002.5646.

[6] PAULA LEI, Q.; LAMB, D. H.; HELLER, R. K.; SHANNON, A. G.; RYALL, R. and CASH, P. 'Kinetic studies on the rate of hydrolysis of N-ethyl-N′-(dimethylaminopropyl)carbodiimide in aqueous solutions using mass spectrometry and capillary electrophoresis'. *Anal. Biochem.* Nov. 2002, vol. 310, no. 1, pp. 122–124. Available from: https://doi.org/10.1016/S0003-2697(02)00276-2.

[7] GILLES, M. A.; HUDSON, A. Q. and BORDERS, C. L. 'Stability of water-soluble carbodiimides in aqueous solution'. *Anal. Biochem.* Feb. 1990, vol. 184, no. 2, pp. 244–248. Available from: https://doi.org/10.1016/0003-2697(90)90675-Y.

[8] JACOBSON, B. S. and FAIRMAN, K. R. 'A colorimetric assay for carbodiimides commonly used in peptide synthesis and carboxyl group modification'. *Anal. Biochem.* Jul. 1980, vol. 106, no. 1, pp. 114–117. Available from: https://doi.org/10.1016/0003-2697(80)90125-6.