

# The Effects of Mutations N501Y and E484K in the SARS-CoV-2 Spike Receptor Binding Motif on the Binding Affinity of RBM Fragments for the ACE2 Receptor

Agnieszka Rombel-Bryzek<sup>1</sup>, Danuta Witkowska<sup>2</sup>

<sup>1</sup> Institute of Medical Sciences, University of Opole, Opole, Poland

<sup>2</sup> Institute of Health Sciences, University of Opole, Opole, Poland

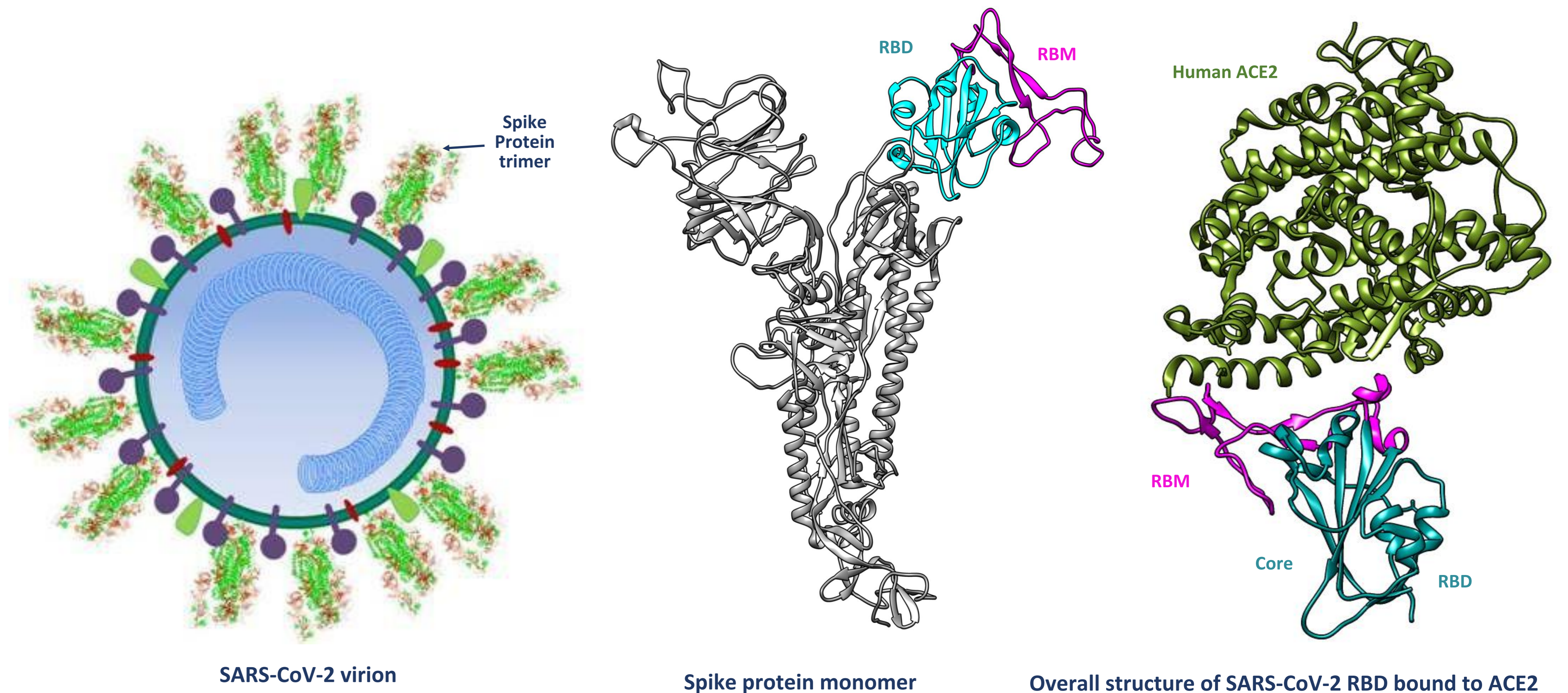


## Abstract

COVID-19 is an infectious disease caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). A critical, initial step for SARS-CoV-2 to enter the host cells is specific interaction the SARS-CoV-2 virus spike protein receptor binding motif (RBM) with angiotensin-converting enzyme 2 (ACE2) on the cell surface. The RBM mutations present in the SARS-CoV-2 variants of concern have occurred independently worldwide. The first mutations found in the RBM of SARS-CoV-2 that have the potential to directly affect important features of the virus are N501Y and E484K. Previous studies indicated that these mutations enhance the interaction of the SARS-CoV-2 S protein with the receptor. In addition, some studies show that these mutations may affect the severity of COVID-19, the spread of the virus and the escape from natural or vaccine-induced immunity. Here, we analyse in detail using isothermal titration calorimetry (ITC) the effects of two common RBMs of SARS-CoV-2 mutations, N501Y and E484K, on interaction between RBM and the human ACE2 receptor.

## Introduction

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2, responsible for COVID-19) is enveloped, large, moderately pleomorphic, positive-stranded RNA (+ssRNA) virus belonging to the genus *Betacoronaviruses*. The genome of SARS-CoV-2 encodes the non-structural replicase polyprotein, four structural proteins: spike (S), envelope (E), membrane (M) and nucleocapsid (N), and several additional non-structural proteins called accessory proteins. The spike protein (150 kDa) is a highly glycosylated homotrimer that is distributed on the surface of the virion particles and protrudes radially from the viral envelope, forming a "crown-like" structure. The S-glycoprotein is responsible for the binding of the virion to the host receptor, its fusion with it and entry into the virus. The SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2), an enzyme found on the outer surface of a variety of cells, as their cellular receptor. The coronavirus spike protein contains the receptor-binding domain (RBD). A critical, initial step for SARS-CoV-2 to enter the host cells is specific interaction the SARS-CoV-2 virus spike protein receptor binding domain (RBD) with angiotensin-converting enzyme 2 (ACE2) on the cell surface. The RBD contains a core and an extended loop called the receptor-binding motif (RBM), which interacts directly with ACE2 [1-5]. Mutations in RBD present in SARS-CoV-2 variants of concern, classified by the WHO, have emerged independently around the world. The common mutations found in SARS-CoV-2 RBD, which have direct potential to affect key characteristics of the virus, are N501Y, E484K. The previously studies suggested, that the mutations N501Y (present in the alpha variant) and E484K (present in the beta variant) enhance the interaction of SARS-CoV-2 S protein with receptor. Some studies show that the mutations in RBD that enhance binding to the ACE2 receptor provide facilitate of SARS-CoV-2 immune escape. Moreover these findings suggest that these mutations may directly affect infectivity [3-5]. The aim of this study was the thermodynamic analysis of the interactions between the human ACE2 receptor protein and four selected RBM fragments of SARS-CoV-2: without mutation (wild-type from Wuhan) and with the N501Y and E484K mutations.



## Methods

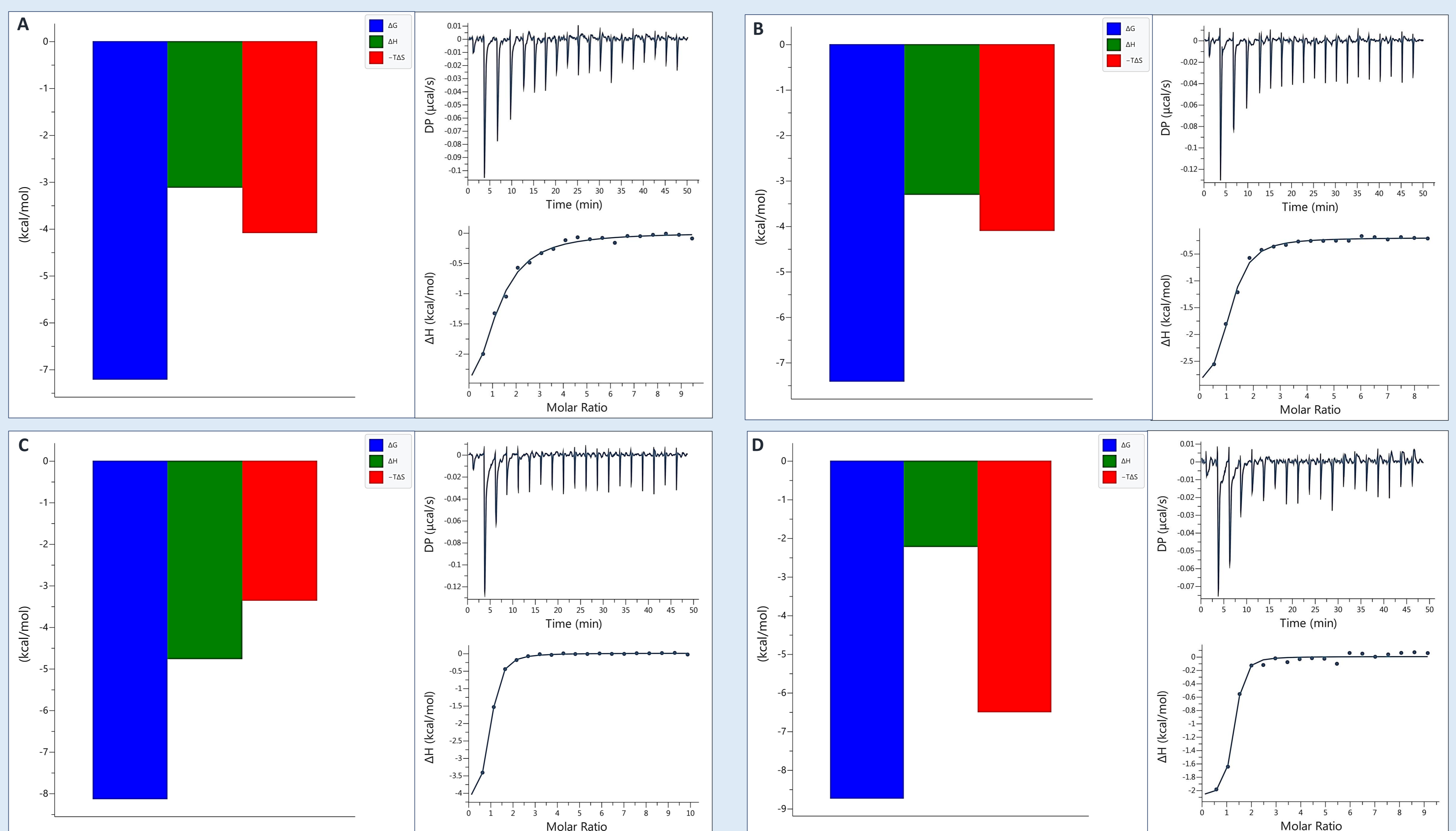
After exhaustive dialysis against PBS buffer, pH 7.4, the interactions of the RBM fragments of SARS-CoV-2 (21 aa) without mutation (wild type from Wuhan, named RBM<sup>WT</sup>) and with the N501Y and E484K mutations (separately and simultaneously, named RBM<sup>N501Y</sup>, RBM<sup>E484K</sup>, RBM<sup>N501Y&E484K</sup>) with the hACE2 receptor were investigated using isothermal titration calorimetry (ITC). For each ITC assay, the RBM fragments and the hACE2 receptor protein were dialyzed against the same buffer and during the same time period (the buffer was exchanged 4-5 times every 12 h) to ensure that all samples were as pure as possible and fit into the correct buffer to avoid heat changes due to buffer mismatch. ITC measurements were performed at 25 °C and pH 7.4 on a MicroCal PEAQ Isothermal Titration Calorimeter. After the instrument was stabilized at 25 °C, 40 mL of RBM fragments buffered solutions were used to titrate 200 mL of ACE2 buffered solutions (concentration initially approx. ten times lower than that of RBM fragments) by 19 consecutive injections with an interval of 150 s between each drop and a stirring speed of 750 rpm (each test was repeated a few times). The reference cell was filled with distilled water.



## Results and Discussion

All analysed RBM fragments bind to the hACE2 protein receptor with a stoichiometry ( $N_{ITC}$ )=1, but with different affinities ( $K_{dITC}$ ). The mutations N501Y and E484K lead to a more than two-fold and almost 6-fold increase in affinity, respectively. The strongest affinity of the RBM fragment for the hACE2 receptor was found in the presence of both mutations simultaneously – a 15-fold increase in affinity compared to the RBM fragment of the wild-type SARS-CoV-2. All studied systems have a similar  $\Delta G_{ITC}$  (the change in free energy), but the highest for the hACE2-RBM<sup>N501Y&E484K</sup> interaction (figure D). Moreover, all interactions are entropy driven, except for the hACE2-RBM<sup>E484K</sup> interaction (figure C), which has the highest enthalpic contribution ( $\Delta H = -4.26$ ) compared to all other systems. This result indicates the thermodynamic stability of the complex (energetically more favourable). Surprisingly, a large entropic penalty is observed for the hACE2-RBM<sup>N501Y&E484K</sup> interaction ( $-T\Delta S_{ITC} = 6.61$ ) (figure D), which could indicate conformational changes. The RBD in the spike protein is the most variable part of the coronavirus genome. Four of five of VOCs (without Delta) contain the N501Y change in the spike glycoprotein which belongs to a set of 6 key amino acid residues essential for binding to hACE2 [6,7]. Many reports have shown that N501Y mutation strongly enhances RBD interaction with human hACE2 [8,9]. In addition, the results suggest that this mutation can increase the stability of the SARS-CoV-2 S protein and virion [10]. On the other hand, previous results indicated that the E484K mutation, which is present in the three VOCs (excluding Delta and Omicron), only slightly increases the affinity for hACE2 [11Lefebvre 2021], which is not consistent with our results. The research will be continued.

	RBM <sup>WT</sup>	RBM <sup>N501Y</sup>	RBM <sup>E484K</sup>	RBM <sup>N501Y&amp;E484K</sup>
$K_{dITC}$ [ $\mu$ M]	4.74 ± 1.45	2.15 ± 0.83	0.81 ± 0.06	0.31 ± 0.08
$N_{ITC}$	0.96 ± 0.13	0.973 ± 0.14	0.743 ± 0.03	0.91 ± 0.02
$\Delta H_{ITC}$ [kcal/mol]	-3.4 ± 0.68	-3.29 ± 0.70	-4.26 ± 0.24	-2.28 ± 0.09
$-T\Delta S_{ITC}$ [kcal/mol]	-3.94	-4.44	-4.06	-6.61



The calorimetric titration isotherms of binding of RBM fragments of (A) wild-type SARS-CoV-2, (B) N501Y mutation, (C) E484K mutation and (D) N501Y & E484K mutations to the hACE2 receptor protein

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