Limitation of tuning the antibody-antigen reaction by changing the value of pH and its consequence for hyperthermia

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Distribution of the isoelectric point (pI) was calculated for the hypervariable regions of Fab fragments of the antibody molecules, which structure is annotated in the structural antibody database SabDab. The distribution is consistent with the universal for all organisms dividing the proteome into two sets of acidic and basic proteins. It shows the additional fine structure in a form of the narrow-sized peaks of pI values. This is an explanation why a small change of the environmental pH can have a strong effect on the antibody-antigen affinity. To show this, a typical enzyme-linked immunospecific assay experiment for testing the reaction of goat anti-human IgA antibodies with human IgA immunoglobulins of saliva as antigens was modified in such a way that Fe₃O₄ magnetic nanoparticles were added to PBS buffer. The magnetic nanoparticles were remotely heated by the radio frequency magnetic field providing the local change of temperature and pH. It was observed that short times of the heating were significantly increasing the antibody-antigen binding strength while it was not the case for a longer time. The finding discussed in the study can be useful for biopharmaceuticals using antibodies, the immunoassay techniques as well as for control over the use of hyperthermia.

Keywords: antibody-antigen complex isoelectric point magnetic nanoparticles magnetic hyperthermia Raman spectroscopy.

The recent increase of research activity on determining antibody structure and function is partly due to the enormous pressure from the new trends in biopharmaceutical industry using antibodies (1–4), from the growing importance of antibody therapy and disease diagnosis (5–8) and also from the developing new

immunoassay techniques (9, 10). Controlling the antibody-antigen affinity (binding strength) becomes the crucial point of the undergoing research. The antigen-combining site in the antibody (paratope) which binds antigenic determinant (epitope) of the antigen is represented by six hypervariable segments of the antibody molecule, three on the light chain denoted by L1–L3 and three on the heavy chain denoted by H1–H3. These hypervariable segments are known as complementarity-determining regions (CDRs) (11). In contrast, the regions outside of the CDRs are represented by constant amino acid composition and they are not considered to form bonds with antigen. Thus, the charge acquired by CDR side chains at a given pH value becomes one of the most important factors for binding with antigen. It is expected that antibodies should follow some selectional constraints for their isoelectric point (pI) value as other proteins do. In article by Kiraga *et al.* (12), it has been shown that the distribution of pI of proteins in a proteome is universal for all organisms. It shows bimodal behaviour which divides the whole proteome into two sets of acidic and basic proteins with respect to their function and only a small fraction of proteins with pI close to 7.4 can be found (13–19). The proteins lose their high level structure stability at the value of pH close to their pI as well as their solubility becomes minimum at pI (20). In particular, the effect of the change of pI by replacing Asp and Glu residues on ribonuclease Sa was discussed in (20). The most stable and active proteins are in the evolutionary distinguished range of pH close to 7.4. Proteins activity is often represented by a bell-shaped curve depending on pH with the possible several maxima determining the optimum pH values (21). In article (21), a detailed study can be found on the pH of maximal activity and its correlation with the pH of maximal stability based on a set of 310 proteins.

In the case of antibodies, it was shown experimentally the increased precipitation activity of antibody to antigen in such pH stability region, *e.g.* Gould *et al.* (22) observed the increased precipitation for immunoglobulin solution and antigen. The pH activity of antibody-antigen reaction and its control by magnetic hyperthermia is the subject of this study. In the following, the distribution of pI values for known CDR sequences was presented to explain the effect of magnetic hyperthermia on antibody-antigen binding strength in the case of the enzyme-linked immunospecific assay (ELISA) (23) modified by using the magnetite nanoparticles heated by RF magnetic field. This modified version of ELISA may be considered as one of the methods that lead to nano-ELISA.

Experimental and Methods

Immunoassays

The magnetically modified method for the reaction of goat anti-human IgA antibodies with human IgA immunoglobulins of saliva as antigens consisted of the following three stages, briefly:

Antigen solution. Pooled saliva was prepared and rotated in centrifuge to get supernatant. Three hundred microlitres of supernatant was added to 30 ml of borate buffer pH 8.2 (for the Raman spectroscopy PBS was used instead) to prepare antigen substrate. Durham tubes were filled with 100 µl of the substrate and incubated overnight in the refrigerator. Tubes were washed four times with PBS, 200 µl of PBS with 1% casein was added to block space free of antigens and they were incubated for 1 h at room temperature. Next, Durham tubes were washed twice with PBS and tween.

Antibody-Fe₃O₄ solution. The goat anti-human IgA peroxidase conjugated (Jackson ImmunoResearch) was prepared in 10 ml of PBS with 1% casein and tween (final dilution 1/5,000). Next, 0.5 ml of magnetic nanoparticles suspension (0.1 g of Fe₃O₄ dispersed in 10 ml of distillate water) was added.

Antigen-antibody reaction and detection. One hundred microlitre of antibody-Fe₃O₄ solution was added to antigen coated Durham tube and the sample was subject to magnetic hyperthermia, next washed four times with PBS and tween. The control Durham tubes which were not exposed to hyperthermia were incubated at room temperature and next washed four times with PBS and tween. For antibody-antigen complexes detection ortho-phenylenediamine and H₂O₂ in citrate buffer (pH 4.9) were used.

Magnetic nanoparticles synthesis

The Fe₃O₄ nanoparticles were synthesized according to Massart (24) method. All chemicals are of analytical reagents and they are used directly without further purification. In the beaker, water solutions of 137 mM of FeSO₄·7H₂O and 274 mM of FeCl₃·6H₂O were prepared. Ultrasonic bath (300 W, 35 kHz) was applied. Next, the solution was stirred mechanically at 1,200 rpm while being heated up to 343 K and 130 mM of NH₄OH was added dropwise, stirred by additional 30 min. The final value of pH exceeded 10. Next, nanoparticles were magnetically decanted, washed 5–7 times with distillate water. The value of pH decreased to 7. The nanoparticles were dried under vacuum for 500 min in 55°C. The black powder consisting of the magnetic nanoparticles with dimension of ~10 nm was obtained.

Measurement characterization

The following techniques were used to investigate on antibody-antigen reaction product:

- Absorbance of the antibody-antigen complex (peroxidase activity) was recorded with the help of UV -

2,450 Shimadzu spectrophotometer. In addition, DYNATECH MR5000 was used.

- Raman spectra were recorded in a backscattering configuration on Renishaw InVia spectrometer using 532 nm diode laser (diode-pumped, frequency-doubled Nd:YAG) and high-power near infrared diode laser (785 nm). The spectra were gathered with 0.1–10% of laser power (0.29–29 mW).
- Radio frequency (~100 kHz) magnetic field generator was used for remote heating of the magnetic nanoparticles. The device was designed for 2 ml microcentrifuge type. The sample was put directly in the centre of a copper coil (six turns). The system was cooled by water flow through the interior of the coil. The magnetic field induced in a coil was equal to 24 mT. The values of temperature were measured with the help of thermographic camera placed above the sample.

CDR sequences characterization

Amino acid sequences representing CDRs (L1–L3, H1–H3) were downloaded from publicly available Structural Antibody Database (SabDab)—downloading address <http://opig.stats.ox.ac.uk/webapps/sabdab>. In July 2015, this database contained 2,103 annotated antibody structures. The Chothia antibody numbering scheme (25) was applied to the selected amino acid sequences. In publication (26), it was stated that the antibody and antigen structures annotated in SabDab are highly redundant in terms of sequence. Therefore, a non-redundant subset of Chothia-defined CDRs was downloaded with an additional database option to search only CDRs from antibodies paired with an antigen. The whole set of Chothia-defined CDRs was additionally downloaded for comparison purposes.

For each CDR sequence the value of pI was calculated. In the algorithm for the estimation of pI the charge *C* acquired by CDR at a particular value of pH was calculated according to the following formula

$$C = C_R + C_D + C_C + C_E + C_H + C_K + C_Y \quad (1)$$

where the symbols on the right side of the Eq. 1 denote the summed charges of amino acids in different amino acid groups R, D, C, E, H, K, Y, respectively. The pK values necessary to calculate the charge on a protein at a given pH have been taken from (27, 28). The same pK values were used by one of us in article (12).

The histogram of the pI value for L1–L3, H1–H3 taken from a sequence-non-redundant subset of Chothia CDRs and the whole set of Chothia-defined CDRs have been plotted in Fig. 1. Only complete CDRs were under consideration.

Results and Discussion

The distribution of pI values for CDRs, which has been shown in Fig. 1, shares the universality feature discussed for proteome in article (12) that the value of pI of proteins composed of naturally occurring amino

Fig. 1 Distribution of pI for the set of all complete Chothia-defined CDRs taken from the structural antibody database SabDab (July 2015) and a sequence-non-redundant subset of them. Total number of sequences in the non-redundant subset is equal to 2,515 to which contribute 414 L1 sequences, 335 H1 sequences, 285 L2 sequences, 453 H2 sequences, 456 L3 sequences, 572 H3 sequences.

Fig. 2 Distribution of amino acids in a sequence-non-redundant subset of Chothia-defined CDRs. Bars in black represent charged amino acids used in Eq. 1 to calculate the charge acquired by CDRs for a given pH value.

acids tends to belong to two sets of acidic or basic proteins. Figure 1 suggests some additional fine structure of the pI values for CDRs in each set, which is represented by several narrow-sized peaks. The calculated average length of CDRs contributing to these peaks is only 9.12 amino acids. The amino acid composition of the analysed CDRs has been shown in Fig. 2. It can be observed the specific usage of charged amino acids (bars in black) which in the case of Y exceeds 10%. At the same time, the usage of the neutral amino acid group S exceeds 15%. This means that the emergence of new CDRs in the database SabDab will not introduce significant changes in the distribution of pI unless the usage of amino acids shown in Fig. 2 is changed.

The observed narrow-sized peaks separate the regions of pH values that are associated with the function of the particular CDRs. The largest of them with pH close to 7.4 is of the order of magnitude of pH 1

and is characteristic for the whole proteome, not only antibodies. In Figs 3–5, there have been presented histograms of pI usage for a sequence-non-redundant subset of Chothia CDRs separately for L1–L3, H1–H3. The ratio of the number of acidic hypervariable segments to basic segments on the light chain and heavy chain is the following:

$$\begin{aligned} (\#L1)_{\text{acidic}}/(\#L1)_{\text{basic}} &\approx 1.1, & (\#H1)_{\text{acidic}}/(\#H1)_{\text{basic}} &\approx 5.6, \\ (\#L2)_{\text{acidic}}/(\#L2)_{\text{basic}} &\approx 1.6, & (\#H2)_{\text{acidic}}/(\#H2)_{\text{basic}} &\approx 4.0, \\ (\#L3)_{\text{acidic}}/(\#L3)_{\text{basic}} &\approx 4.12, & (\#H3)_{\text{acidic}}/(\#H3)_{\text{basic}} &\approx 9.4. \end{aligned}$$

These numbers suggest the strong asymmetry for the benefit of the acidic sequences. The calculated statistical significance between the groups of the heavy and light chains using a standard 95% confidence level shows that the heavy chains are more likely to be acidic than the light chains (a *t*-statistic yields the value of $|t| > 1.96$ where $|t| = 11.58$).

The detailed investigation on the possible amino acid usage correlation between L1, L2, L3, H1, H2 and H3 is out of the scope of this study. The results in Figs 3–5 have been presented to show that such correlation can exist. The short range intervals of pH interrupted by pI peaks observed in Fig. 1 suggest that even small environmental change of pH can influence the formation of the antibody-antigen complex. It can be expected from Fig. 1 that antibody activity will be greatest close to pH 7.4. The observation of the increased antibody activity in this region of pH values is known in literature for a long time, *e.g.* (22).

In Fig. 6, it has been shown the dependence of UV-Vis absorbance for the reaction of goat anti-human IgA antibodies with human IgA immunoglobulins of saliva as antigens where the reaction took place in the wells of ELISA plate. The antigen solution in Fig. 6 was diluted 20 times. It is evident the step-like shape of the measured absorbance with the largest values at pH close to 7.4. The values of pH in the interval from 5.5 to 8.5 are associated with the enhanced reactivity of antibody and antigen. The results are consistent with the expectations which we could derive from Fig. 1 as well as with the results for the increased precipitation activity of antibody to antigen published in Ref. 22.

To verify the suggestion that the small change of pH can affect the formation of the antibody-antigen complex, the ELISA experiment from Fig. 5 was modified by insertion of 0.1% water solution of Fe₃O₄ magnetic nanoparticles into PBS and next the nanoparticles were heated remotely by the applied RF magnetic field. Durham tubes were used in this version of ELISA instead of microtiter plates because of the requirements from hyperthermia device.

Three types of experiments were performed, the first one for antibody-antigen complex formation, the second one for antibody-antigen reaction in the presence of magnetic nanoparticles and the third type is the second one with the magnetic hyperthermia applied. The experiments are shortly denoted as Ab/Ag, Ab/Ag/MNP and Ab/Ag/MNP^h. In Table I, absorbance at wavelength of light $\lambda = 495$ nm for these three types of antibody-antigen reactions has been presented. The antigen solution under consideration was diluted 100 times.

Fig. 3 Distribution of pI for L_1 and H_1 .

Fig. 4 Distribution of pI for L_2 and H_2 .

The experiments were carried out for 30–60 min, respectively. In both cases, temperature was measured with the help of thermographic camera placed above the sample. Its value was not changing after reaching 35.5°C. The RF magnetic field generator was designed for heating a single sample in a given time interval and it was the direct cause of choice at least 30 min for one measurement to ensure the same experimental conditions.

In Table I, it can be noticed that addition of the magnetic nanoparticles increases the value of absorbance. The explanation for this observation can be that insertion of magnetic nanoparticles increases the

effective reaction area for the antibody-antigen complex formation. This is possible due to the positive charge, which is acquired by the surface of the Fe_3O_4 magnetic nanoparticles in PBS (pH 7.4)—point of zero charge for magnetite nanoparticles in water solution is around pH 7.9. Similar idea of using magnetic particles can be found in article (10) for the nano-ELISA assays where the micro-magnetic beads were modified with monoclonal antibody of the target protein p53.

The increase of absorbance observed for ELISA with magnetic nanoparticles can be also associated with pH buffering property published in article (29),

Fig. 5 Distribution of pI for L3 and H3.

Fig. 6 The effect of pH on binding activity of goat anti-human IgA antibodies to human IgA immunoglobulins of saliva as antigen has been shown with the help of the UV-Vis absorption spectroscopy where the absorbance of the solution versus pH at wavelength $\lambda = 495$ nm is plotted. The dashed line is guide for eyes only.

where it was shown that the presence of iron oxide magnetic nanoparticles in a water solution is sufficient to change pH value of the solution close to the nanoparticle surface even by pH 0.5 both at acidic and basic range of pH values.

Surprising in Table I could be that heating the magnetic nanoparticles for 30 min increased the absorbance almost twice. On the other side, heating the nanoparticles for 60 min decreased the absorbance by two orders of magnitude. The decrease of pH with the increased temperature could be the explanation for observing the change of the absorbance. In article (30), it was found experimentally the relationship between temperature and albacore blood pH which

Table I. Absorbance for different heating times

	Ab/Ag	Ab/Ag/MNP	Ab/Ag/MNP ^h
After 30 min	0.144	0.208	0.252
After 60 min	0.173	0.25	0.003

UV-Vis absorbance at $\lambda = 495$ nm for three types of ELISA experiment: Ab/Ag representing antigen-antibody complex in PBS, Ab/Ag/MNP the same as Ab/Ag but PBS contains Fe_3O_4 magnetic nanoparticles, Ab/Ag/MNP^h the same as Ab/Ag/MNP but the magnetic hyperthermia is applied.

shows the linear dependence on temperature T such as $\text{pH} = a - 0.016 T$ where a depends on arterial and venous CO_2 pressure. The strong decrease of absorbance observed in Table I in the case of magnetic hyperthermia applied for 60 min suggests that the resulting large change in pH produced conditions unfavourable for the antibody-antigen reaction.

The effect of magnetic nanoparticles on antibody-antigen binding process as well as the effect of the remote heating them by RF magnetic field was examined also at molecular level with the help of the Raman spectroscopy. Raman spectroscopy becomes a powerful tool for identifying organic components, *e.g.* to diagnose periodontitis (31).

In view of the very complex structure of the Raman spectrum only the band ranges characteristic for Fe_3O_4 magnetic nanoparticles have been shown in Fig. 6. These bands are around 540 and 668 cm^{-1} which confirm the presence of magnetite nanoparticles (32, 33). The spectrum of magnetite nanoparticles strongly depends on laser power (33) and raising the power can lead to the appearance of the peaks characteristic for hematite phase, like the one around 1,322 cm^{-1} . In the case of the antibody-antigen solutions, the small

Fig. 7 Collected fragments of the Raman spectra for three types of ELISA experiments Ab/Ag, Ab/Ag/MNP, Ab/Ag/MNP^h and Fe₃O₄ magnetic nanoparticles in PBS (denoted as MNP) have been shown.

intensity of their Raman signals requires laser power even by two orders larger than the one appropriate for magnetite nanoparticles and laser power 29 mW was used in Fig. 6. This explains the appearance of signals from hematite in the spectra.

The plots representing the experiments Ab/Ag/MNP and Ab/Ag/MNP^h which were carried out, respectively, for 30–60 min show the qualitative differences. It is evident the destructive role (Table I) of magnetic hyperthermia applied for 60 min where some peaks disappear. In contrast, the Raman spectra for Ab/Ag/MNP and Ab/Ag/MNP^h carried out for 30 min are qualitatively the same. The latter property supports the observation of qualitatively different absorbances in Table I for magnetic hyperthermia.

Conclusions

The pI distribution of CDRs, introduced in this study, suggests the evolutionary given pattern of pH stability for antibody-antigen complex formation. It was shown the possibility to control such complex formation by the change in environmental pH. The finding could have practical significance. In particular, the recent investigation on antibody therapy shows that shifts in the value of pI of around ± 1 can produce measurable changes in tissue distribution and kinetics (1). The magnetic hyperthermia coupled with the antitumour immunity can significantly enhance the latter one. The concept that the immune system has a role in controlling cancer comes back in the recent article (8).

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Conflict of Interest

None declared.

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