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# Phage display derived therapeutic antibodies have enriched aliphatic content: Insights for developability issues

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## Abstract

Phage display is one of the most widely used technology for antibody discovery and engineering. Number of therapeutic antibodies derived from phage display increases rapidly due to its ease of use and ability to control antibody sequence information. Although there are numerous antibody candidates as promising therapeutics, most of them fail at later stages of development due to undesired biophysical properties. Antibody candidates with poor properties should be prevented or improved in early development phases to minimize enormous loss of time and resources. In this study, we showed that phage display derived therapeutic antibodies show higher self-interaction and polyspecificity compared to non-phage display derived ones. To identify molecular determinants behind this, physicochemical properties of CDR regions of 137 therapeutic antibodies were analyzed. We found multiple significant differences in both heavy and light chain CDR regions. Most profoundly, aliphatic content of HCDR3, HCDR2, and LCDR3 regions were enriched in phage display derived antibodies compared to non-phage display derived ones. Physicochemical determinants documented here seem to play important roles in polyspecific and aggregation-prone natures of antibodies which should be avoided in early development phases.

**KEYWORDS**

antibody, complementary determining regions, phage display, physicochemical properties

## 1 | INTRODUCTION

Within the last decade, monoclonal antibodies have gained importance due to being the fastest growing class of biopharmaceuticals.[1] Currently, more than 80 antibody-based drugs have been approved and become commercially available in many countries, and many more are already in advanced clinical trials.[2–7] Previously, antibodies were chosen based on their functionality, meaning how specific and strong they bind to their target epitopes and how they exhibit desired functions such as antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cellular lysis, neutralization of infectivity, antibody opsonization, and phagocytosis.[8] However, this traditional approach leads to unsuccessful candidates with undesired biophysical characteristics; therefore, majority of those preclinical/clinical candidates are not on the market due to their developability issues. Hence, there are numerous possible drug candidates with the potential of further development, and there should be a suitable selection criterion which are examined under the aspect of developability.[9] Antibody candidates with good developability properties should harbor the following biophysical characteristics: (1) having high expression levels, (2) high solubility required to reach high concentration enough for low volumes of dosage, low viscosity to ease the administration process, appropriate pK properties, (3) low poly-specificity and minimum immunogenicity, (4) being chemically stable under in vivo conditions, and (5) low self-interaction.[10,11] The combination of many different biophysical and physicochemical properties define desired candidates; therefore, assessment of the antibody developability is a multi-staged approach where the undertaken trade-offs to be considered carefully.[12]

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Developability issues are generally related with intrinsic and extrinsic physicochemical properties. Antibody drugs are not orally active and therefore require injection at high concentrations.[13] One of the most challenging parts of antibody production is to manufacture antibodies in higher yields and formulate them stably in liquid form. Proteins are prone to self-interaction at higher concentrations which brings along the risk of aggregation; therefore, it is critical to determine aggregation tendency at the earlier stages.[14–16] There are many factors affecting the protein aggregation such as hydrophobicity, electrical charge, and propensity.[17,18] Antibodies with poor solubility properties should be immediately engineered and/or eliminated in the earlier development phases to reduce the burden of time and resources; meanwhile, this renders the minimization of possible risks of downstream processes.[19] In such cases, protein engineering and/or directed evolution approaches can be implemented to obtain advanced candidates in early development phases.[20,21] Nowadays, there are substantial high-throughput experimental methods and in silico tools available for screening and improvement of these candidates at earlier stages which speeds up the entire process as well as increases the product quality.[22] One of the most commonly used directed evolution method is phage display technology which is recently awarded 2018 Nobel prize in Chemistry. Phage display allows the presentation of peptide and protein libraries "displayed" on the surface of filamentous phage.[23] Antibodies are subsequently selected based on affinity and/or biophysical properties. This technique is used in a variety of applications including protein engineering, affinity maturation, and drug discovery. Phage display libraries containing several billion variants (in the range of $10^6$ to $10^{11}$) can be constructed and screened simultaneously.[24] This makes it possible to screen combinatorial antibody libraries for directed evolution approaches.[25]

There are four kinds of antibody phage libraries: immune, naïve, semi-synthetic, and synthetic libraries.[26] Immune, naïve, and semi-synthetic libraries are mostly used to discover novel binders for a specific target, whereas synthetic libraries are designed custom-based mostly for antibody engineering and affinity maturation approaches.[27,28] Synthetic/semi-synthetic libraries are usually based on diversity in CDRs (complementarity determining regions) of antibodies, which are responsible for antigen binding and are naturally diverse regions. There are six CDR loops in each antibody, three on heavy chain, three on light chain. Most diversity is naturally found in CDR3s of heavy and light chains due to multiplicity of V(D)J combinations and inclusion of N/P nucleotides, whereas the other four CDRs show limited variation (encoded by only V-gene segment).[29] In addition to VDJ combinations, HCDR3 shows variability in length, and this makes HCDR3 the most diverse region important for defining antibody specificity and affinity.[29,30] HCDR3 has therefore been the initial target for introduction of diversity in synthetic libraries as it has been shown in many different prior studies.[31,32] Besides affinity, composition of CDR loops have a significant influence on the specificity and biophysical characteristics of an antibody.[25] This is important to take into consideration when creating diversity in synthetic/semi-synthetic libraries, as the natural selection in the immune cells is absent. Generally, the use of the most hydrophobic amino acids is kept at low levels (0%-25% likelihood of a hydrophobic amino acid at any given randomized position). This is usually preferred to lower the overall hydrophobicity of the CDRs, thereby reducing the risk of aggregation, and at the same time preserving favorable hydrophobic interactions with antigen.[33,34] Additional favorable contributions to binding documented for tyrosine, serine, and glycine can be taken into account in the design of CDR synthetic libraries.[35,36] Positively charged amino acids are usually kept at very low frequencies for CDRs, because a high frequency of those amino acids have been linked to low specificity and increased nucleic acid binding of the antibodies.[35,36] However, detailed physicochemical composition analysis of therapeutic antibody CDRs associated with their developability properties has not been well defined yet.

In this study, sequence and biophysical data of several therapeutic antibodies were statistically analyzed as summarized in Figure 1, and certain physicochemical patterns were documented for phage display derived antibodies. We found that phage display derived therapeutic antibodies have higher self-interaction and polyreactivity properties compared to non-phage ones. To understand molecular reason(s) behind this, we analyzed physicochemical characteristics of CDR regions of 137 therapeutic antibodies, 34 of which was derived by phage display. We showed that aliphatic contents of HCDR3, HCDR2, and LCDR3 of phage display derived antibodies are significantly higher than other antibodies. Physicochemical features of these regions and other CDRs documented here might explain disadvantageous properties of phage display derived antibodies. Therefore, synthetic/semi-synthetic libraries of antibody CDRs should be designed considering certain physicochemical properties reported here.

## 2 | MATERIALS AND METHODS

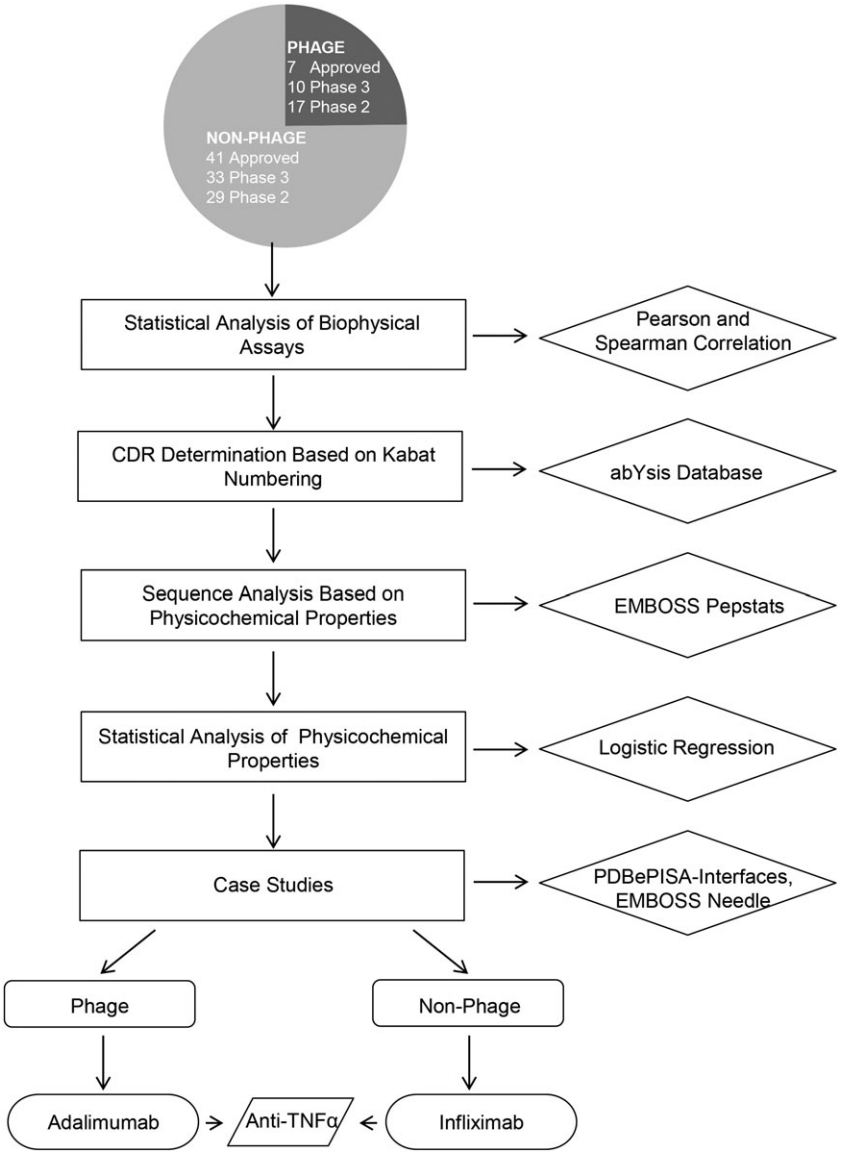
### 2.1 | Data

Data for 137 different antibodies were collected from the study of Jain, et al.[37] The data includes light and heavy chain variable region sequence information and 12 separate biophysical characterization assay results of those antibodies. At the time of the study, 46 of those 137 antibodies were already approved by FDA, 46 of them were in phase II trials, and 43 were in phase III trials in various countries including USA and Europe. Based on human light chain properties, 124 of them have kappa (κ), and the remaining 13 have lambda (λ) light chains. Although 34 of these antibodies are derived from phage display (phage), 103 of those are generated with other techniques, mostly hybridoma (non-phage). Biophysical assay results were used to evaluate developability properties of those antibodies, via assessment of yield, stability, hydrophobic interactions, self-interaction, and polyreactivity.

### 2.2 | Statistical analysis of biophysical assays

Data analysis software SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA) was used to run a descriptive (mean) and inferential (Pearson correlation) analysis. Pearson correlation *P*-values and coefficients were reported to show the relation between two variables. One variable was phage/non-phage class, and the other variable is one of 12 different biophysical characterization results. This analysis showed overall

**FIGURE 1** Work-flow of the study




significance of phage/non-phage classifications based on biophysical characteristics of antibodies. The pair(s) of variables with positive correlation coefficients and *P*-values below 0.050 tend to increase together. For the pairs, with negative correlation coefficients and *P*-values below 0.050, one variable tends to decrease, whereas the other increases. For pairs with *P*-values greater than 0.050, there is no significant relationship between the two variables. Pairs with *P*-values below 0.01 represent highly significant relationship.

## 2.3 | Data for physicochemical properties

$V_H$ (heavy chain variable region) and $V_L$ (light chain variable region) sequences of all antibodies were obtained from Jain, et al.[37] Sequences were classified under two categories as antibodies derived from phage display (phage) or not (non-phage). Amino acid sequences of $V_H$ and $V_L$ domains of all antibodies are aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). CDR sequences of $V_H$ and $V_L$ domains were determined according to the Kabat numbering scheme for further analysis. To determine protein properties of CDRs, EMBOSS Pepstats was used (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/).[38] Table 1 shows physicochemical properties of amino acids used in this study. In addition to properties listed in Table 1, frequency of each individual amino acid is also analyzed. Because some CDRs, especially HCDR3, have different lengths, all properties were weighted by CDR length (number of amino acids/length). Natural repertoire values were obtained from abYsis database (http://www.bioinf.org.uk/abysis3.1/), and amino acid frequency values of *Homo sapiens* and *Mus musculus* were used based on Kabat numbering for all CDR regions.

## 2.4 | Statistical analysis of physicochemical properties

A linear regression model was implemented to examine the relationship between phage/non-phage class and the chosen physicochemical property weighted by CDR length. Forward stepwise regression model was done for each CDR region (HCDR1/2/3, LCDR1/2/3), and

**TABLE 1** Physicochemical classifications of amino acids used in this study

| Property | Residues |
|---|---|
| Tiny | A, C, G, S, T |
| Small | A, B[a], C, D, G, N, P, S, T, V |
| Aliphatic | A, I, L, V |
| Aromatic | F, H, W, Y |
| Non-polar | A, C, F, G, I, L, M, P, V, W, Y |
| Polar | D, E, H, K, N, Q, R, S, T, Z[b] |
| Charged | B[a], D, E, H, K, R, Z[b] |
| Basic | H, K, R |
| Acidic | B[a], D, E, Z[b] |

[a]B = Asn or Asp.
[b]Z = Gln or Glu.

*P*-values of less than 0.05 were considered significant using SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA). Box plots with corresponding *P*-values, maximum, minimum, median, first, and third quartile values were generated for visualization.

## 2.5 | Case studies

A case study was investigated to represent our findings. Two commercially available therapeutic monoclonal antibodies targeting the same antigen, TNFα (tumor necrosis factor alpha), were chosen for the case study: adalimumab and infliximab. 3D protein structures in complex with TNFα were extracted from The Protein Data Bank (PDB) for adalimumab (Humira, PDB ID: 3WD5)[39] and infliximab (Remicade, PDB ID: 4G3Y).[40] Molecular visualization of structures was done by the aid of PyMOL (The PyMOL Molecular Graphics System, Version 2.2 Schrödinger, LLC). Hydrogen bonds (H-bonds), salt bridges, and hydrophobic interactions within interfacing residues of two antibodies and TNFα were defined by PDBePISA-Interfaces tool (http://www.ebi.ac.uk/pdbe/pisa/)[41] and further confirmed by PyMOL 2.2 software. Heavy and light chains of adalimumab and infliximab were aligned through EMBOSS Needle Pairwise Sequence Alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).[38]

# 3 | RESULTS

## 3.1 | Phage display derived antibodies show higher self-interaction and poly-reactivity

Dataset of 137 clinical-stage antibodies includes sequence information of variable regions ($V_H$ and $V_L$) and 12 biophysical characterization assay results.[37] Although 34 of these antibodies are derived (discovered and/or engineered) by phage display, the remaining 103 of these are generated with other techniques, mostly hybridoma (non-phage display). Biophysical assay results were used to evaluate developability properties of antibodies by clustering them into five major classes: (1) yield, (2) stability, (3) hydrophobic interactions, (4) self-interaction, and (5) poly-reactivity (Table 2).

**TABLE 2** Pearson correlation analysis for pairs of phage/non-phage classes and each biophysical characterization values [Color figure can be viewed at wileyonlinelibrary.com]

| | HEK Titer | $T_m$ | Accelerated Stability | SGAC-SINS* | HIC Retention | SMAC Retention | CSI-BLI** | AC-SINS** | PSR** | CIC Retention | ELISA** | BVP ELISA** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean (phage) | 131.0 | 71.2 | $7.4\ 10^{-2}$ | 534.3 | 10.3 | 9.8 | $6.6\ 10^{-2}$ | 11.1 | $25.6\ 10^{-2}$ | 9.4 | 3.9 | 7.1 |
| Mean (non-phage) | 152.1 | 71.3 | $6.3\ 10^{-2}$ | 679.0 | 10.3 | 10.0 | $7.6\ 10^{-4}$ | 5.4 | $11.1\ 10^{-2}$ | 9.1 | 1.7 | 3.1 |
| Pearson *P*-value | 0.095 | 0.956 | 0.651 | 0.024 | 0.928 | 0.721 | $8.0\ 10^{-4}$ | 0.007 | $3.3\ 10^{-4}$ | 0.060 | $0.2\ 10^{-4}$ | $2.8\ 10^{-6}$ |
| Pearson correlation coefficient | −0.143 | −0.004 | 0.039 | −0.192 | −0.008 | −0.031 | 0.283 | 0.230 | 0.302 | 0.161 | 0.358 | 0.388 |

Biophysical assays are clustered into different colors based on what they measure (orange: yield, gray: stability, yellow: hydrophobic interactions, blue: self-interaction, green: poly-reactivity).
*P-value < 0.05.; **P-value < 0.01.

To show the relationship between two variables (phage/non-phage and each of 12 biophysical assay value), a Pearson correlation analysis was conducted (Table 2). Although 5 of 12 assays gave a highly significant relationship (*P*-value <0.01), 1 of 12 assays gave a significant relationship (*P*-value <0.05). Highly significant relationships were concentrated into only two biophysical assay classes: self-interaction and poly-reactivity. CSI-BLI[15] and AC-SINS[19,42] represented self-interaction class, and both of them gave highly significant *P*-values and positive Pearson correlation coefficients which show increased self-interaction for phage variable. PSR,[14] CIC retention,[10] ELISA,[43] and BVP-ELISA[13] represented poly-reactivity class, and all of them except CIC retention gave highly significant *P*-values and positive Pearson correlation coefficients which indicate increased poly-reactivity for phage variable. Although CIC retention assay gives insight about poly-reactivity (retention times with polyclonal antibody immobilized column), it is mainly used for solubility determination of antibodies.[10] Among hydrophobic interactions, although SGAC-SINS[44] assay gave a significant *P*-value of 0.024, other two assays did not give any correlation. Overall, biophysical assay results showed higher self-interaction and poly-specificity profiles of phage display derived therapeutic antibodies. To understand the molecular determinants behind this, a physicochemical analysis focused on CDR regions of those antibodies was conducted.

## 3.2 | CDR regions of phage display derived antibodies have distinct physicochemical properties

We argued that physicochemical differences in CDR regions, especially HCDRs, should play a role in undesired biophysical characteristics of self-interaction and polyreactivity. After fitting a linear regression model for physicochemical contents of HCDRs (HCDR1, 2, 3) and LCDRs (LCDR1, 2, 3) of phage and non-phage antibodies, we found multiple significant relationships (Figure 2 and Table 3). Although CDR contents of phage antibodies are mostly human, those of non-phage antibodies can be human, mouse, or humanized. To determine if our findings are not due to human vs mouse differences, we compared CDR amino acid frequency values of phage/non-phage class and natural repertoire of human and mouse (humanized content should theoretically be in between human and mouse frequency) (Figure 3). After this comparison, it turned out that some of significant relationships mostly in light chain CDRs are due to human-mouse CDR differences which are excluded from this study. Aliphatic contents of HCDR3 and HCDR2 were significantly higher in phage display derived antibodies. Most profoundly, frequency of leucine in HCDR3 was much higher than both non-phage antibodies and natural repertoires (Figure 3A). Leucine and isoleucine content seems like the main contributor for enriched aliphatic profile of HCDR3 and HCDR2 regions, respectively. However, glycine showed significant increase in HCDR3 of non-phage antibodies (Figures 2A and 3A). Glycine is a unique, small amino acid with hydrogen as its only side chain. It is already known that tyrosine, serine, and glycine contribute significantly to antigen binding,[35,36] and there is a higher propensity for tyrosine, glycine, aspartate, and phenylalanine in HCDR3 region.[45] Therefore, decreased glycine content of HCDR3 might lead to undesired properties of phage antibodies. Although aliphatic residues are important for protein-protein interactions, they also contribute to self-interaction and aggregation due to their hydrophobic nature. Because aliphatic residues lack H-bond/charge-charge interaction capabilities and definitive geometries (unlike aromatic residues) essential for complementarity, enrichment of them in the binding interface might lead to poly-specificity. HCDR3 is mostly the main player for antibody affinity/specificity/solubility, and this finding lays foundation to the understanding of poly-reactive and aggregation-prone property of phage display derived antibodies.

Hence, only ~30% of CDR residues are usually responsible for antigen binding,[46] rest of exposed CDR residues mostly contribute to other biophysical characteristics of antibodies. Other HCDRs also showed significant relationships (Figure 2A). There are studies showing that more negative HCDR1 make antibodies more aggregation-prone.[34] We found a compatible result that HCDR1 has more negative charge for phage antibodies by having significantly higher aspartate and lower arginine contents (Figures 2A and 3A). More negative HCDR1 might contribute to self-interaction properties of phage derived antibodies. This also shows influence of differential physicochemical characteristics on different HCDR regions on antibody properties. Interestingly, HCDR2 tyrosine content of phage antibodies were significantly lower than those of non-phage antibodies (Figures 2A and 3A). Aromatic residues are usually enriched in antibody paratopes due to their important contributions to antigen binding. Essential roles of aromatic residues in specificity and affinity usually come from their special geometry ($\pi$ systems) and their capabilities of forming multiple non-covalent interactions, respectively.[47] A decrease in tyrosine content might be the cause of phage display derived antibodies showing polyreactivity.

Although light chains are not the usual targets for antibody engineering due to dominating role of heavy chains in antibody binding and characteristics, there is a promise that LCDRs can be engineered like HCDRs for even better biophysical properties, especially for their aggregation resistance.[48] This makes $V_L$s promising therapeutic candidates;[49] therefore, physicochemical properties of LCDRs should be also analyzed in detail. LCDR3 is the most important region for antibody characteristics and it differs between $V_\kappa$ (variable kappa) to $V_\lambda$ (variable lambda) domains.[50] We found that aliphatic content of LCDR3 was increased for phage antibodies (Figure 2B). Because aliphatic amino acids are usually buried in protein core structures for higher stability,[51,52] higher aliphatic residues exposed in LCDR3 region could be an indicative for higher propensity for aggregation. Increase in aliphatic content of LCDR3 mainly contributed by leucine and isoleucine enrichment (Figures 2B and 3B). It is important to note that polar and non-polar classes are also significantly correlated, where there is a non-polar, especially glycine enrichment in LCDR3 regions of phage antibodies (Table 3, Figure 2B). In addition to aliphatic amino acids, non-polar class includes other hydrophobic residues such as aromatic ones and small amino acids such as glycine. There is no significant relationship for aromatic residues, but glycine shows one of the most significant increase in LCDR3 region (Figure 2B). However, there is a different trend in LCDR2 where aliphatic content (especially alanine and leucine) was significantly decreased for phage antibodies. Also, LCDR1 small amino acid content of phage antibodies is higher than that of non-phage class

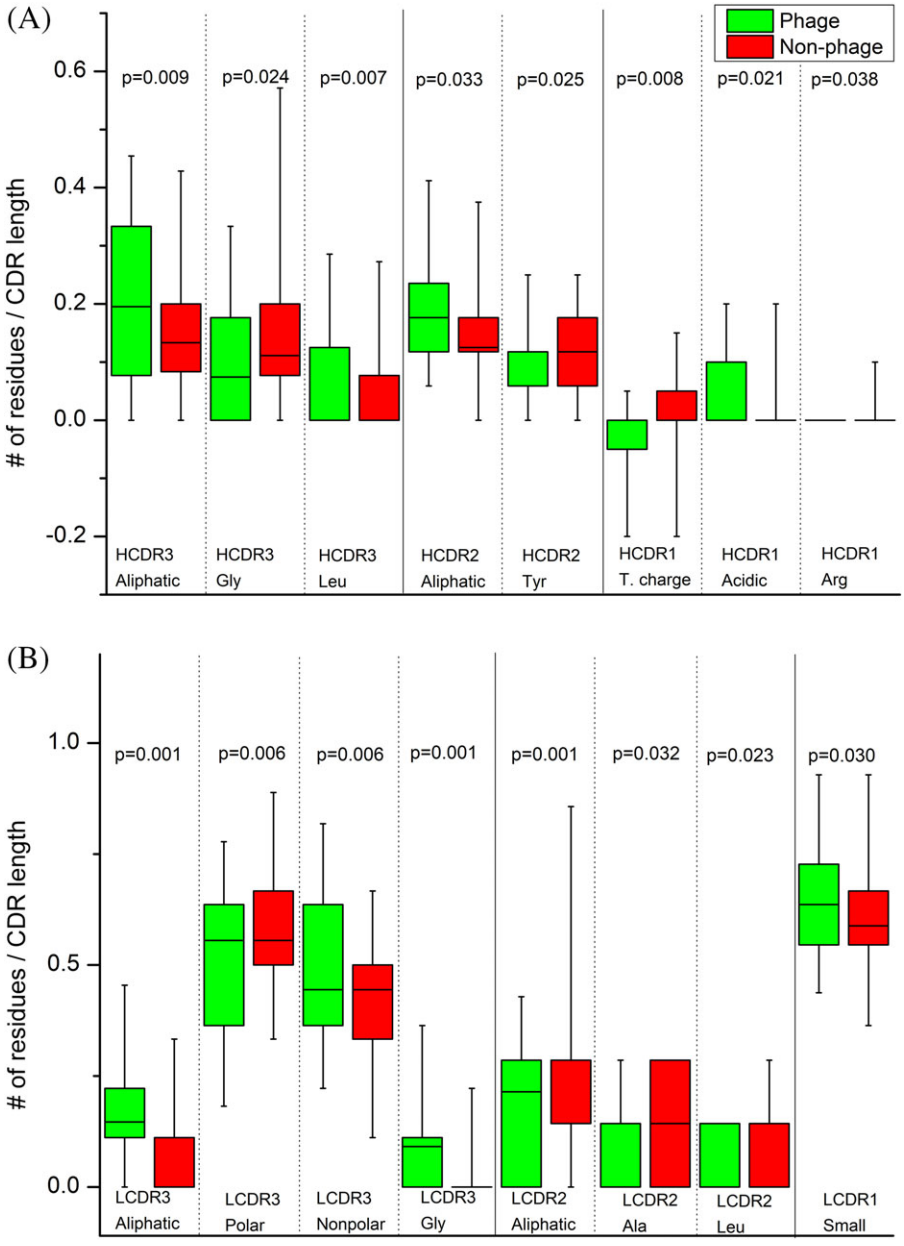



**FIGURE 2** Box plots showing physicochemical distribution for complementarity determining regions (CDRs) of phage and non-phage antibodies. (**A**) HCDR3, HCDR2, and HCDR1 regions; (**B**) LCDR3, LCDR2, and LCDR1 regions. *P*-values from linear regression analysis are reported at the top. Box plots represent maximum, minimum, median, first, and third quartile of the corresponding data [Color figure can be viewed at wileyonlinelibrary.com]

(Table 3, Figure 2B). Among LCDR regions, LCDR3 has been accepted as the only region with possible significant contribution to antibody properties;[48,50] therefore, combinatorial library designs on LCDR is mostly applied on only LCDR3 region. Based on our findings, hydrophobic especially aliphatic residue incorporation into LCDR3 should be minimized when designing combinatorial phage libraries.

### 3.3 | Case Study: adalimumab has a larger interaction surface area and more aliphatic content than infliximab

As a case study, two approved and marketed monoclonal antibodies both targeting same antigen, tumor necrosis factor alpha (TNFα), were chosen. Although adalimumab (trade name Humira®) was heavily engineered by phage display,[39] infliximab (trade name Remicade®) was developed by hybridoma technology.[40] Although starting template of adalimumab was mouse, final form of adalimumab was affinity matured, humanized (chain shuffling), and engineered by phage display system.[53] Both antibodies were used to treat some similar autoimmune diseases by blocking binding interaction of TNFα with its receptors. Adalimumab and infliximab have similar binding affinity to TNFα ($K_D$ = 127 and 44 pM, respectively).[54,55] Adalimumab has less immunogenicity due to its humanization while infliximab is a mouse-human chimeric antibody.[56] Because they bind to the same antigen, a comparison on their CDR content and TNFα interfacing region was conducted to confirm our findings. The first notable thing was the difference in TNFα binding interface area (Figure 4). Although adalimumab has 1269.6 Å (heavy chain: 706.8 Å, light chain: 562.8 Å), infliximab has 989.0 Å (heavy chain: 554.2 Å, light chain 434.8 Å) interface area with TNFα. Also, adalimumab interacts simultaneously

**TABLE 3** Stepwise multiple linear regression analysis with phage/non-phage as a dependent variable performed in all complementarity determining regions of antibodies ($n$ = 137)

| Independent variables | | $\beta$-coefficient[a] | SE[b] | $t$ | $P$-value |
|---|---|---|---|---|---|
| HCDR3 | Aliphatic | −0.841 | 0.316 | −2.661 | 0.009 |
| | Gly | 0.822 | 0.359 | 2.287 | 0.024 |
| | Leu | −1.395 | 0.509 | −2.741 | 0.007 |
| HCDR2 | Aliphatic | −1.023 | 0.475 | −2.153 | 0.033 |
| | Tyr | 1.459 | 0.643 | 2.268 | 0.025 |
| HCDR1 | Total charge | 1.581 | 0.585 | 2.702 | 0.008 |
| | Acidic | −1.631 | 0.700 | −2.33 | 0.021 |
| | Arg | 2.752 | 1.311 | 2.099 | 0.038 |
| LCDR3 | Aliphatic | −1.346 | 0.334 | −4.028 | <0.001 |
| | Polar | 0.800 | 0.285 | 2.805 | 0.006 |
| | Non-polar | −0.800 | 0.285 | −2.805 | 0.006 |
| | Gly | −2.238 | 0.571 | −3.921 | <0.001 |
| LCDR2 | Aliphatic | 0.940 | 0.273 | 3.436 | <0.001 |
| | Ala | 0.708 | 0.327 | 2.168 | 0.032 |
| | Leu | 1.056 | 0.460 | 2.297 | 0.023 |
| LCDR1 | Small | −0.723 | 0.330 | −2.192 | 0.030 |

[a]β-coefficient: standardized regression coefficient.
[b]SE: estimate of SD of β-coefficient.

with two monomers of TNFα trimer, where infliximab forms interaction with only one monomer of the natural trimer form (Figure 4B). Tables 4 and 5 show list of interactions of adalimumab and infliximab with TNFα, respectively. Number of contacts formed is obviously higher for adalimumab. However, number of its HCDR3 contacts (known as the most important region for antigen binding) is much lower than that of infliximab. This might suggest that larger binding area is needed for high affinity/specificity if HCDR3 contribution is at minimum.

According to our statistical analysis, we concluded that HCDR3, HCDR2, and LCDR3 of phage derived antibodies are enriched with aliphatic residues. This trend of aliphatic enrichment is obvious for corresponding CDRs of adalimumab (Figure 4A). Aliphatic residues were heavily enriched alongside HCDR3 contact site residues of

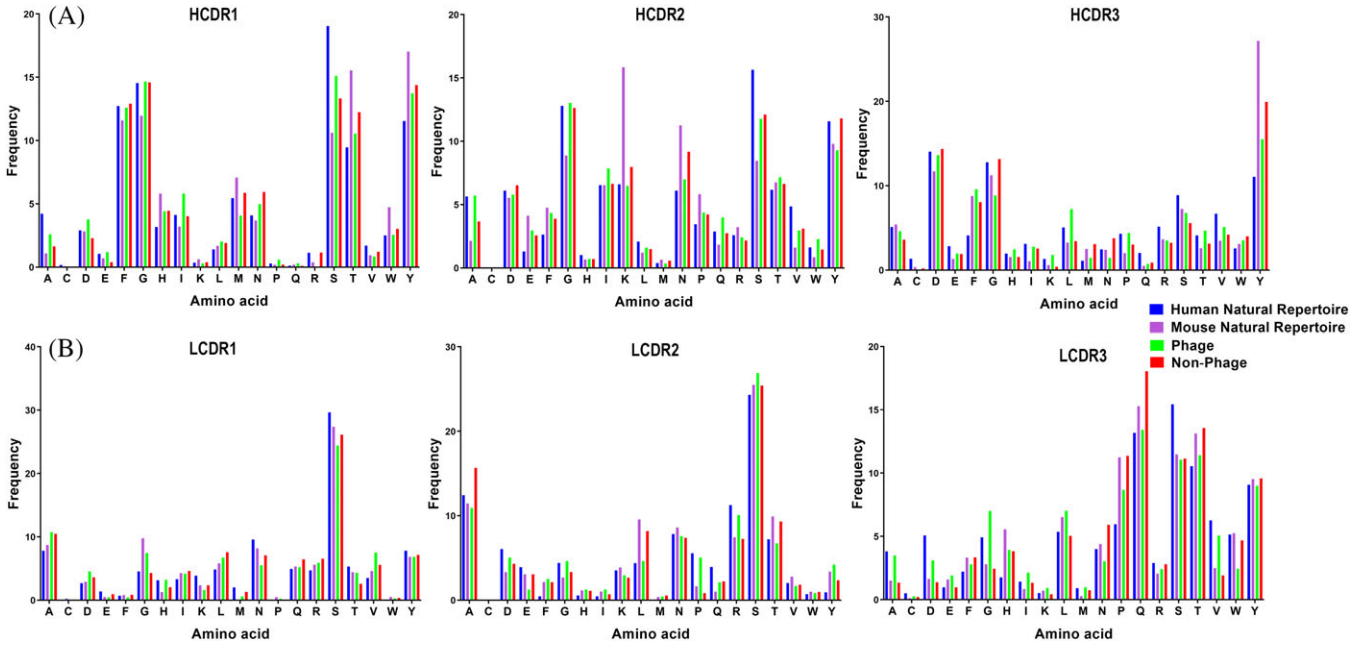



**FIGURE 3** Amino acid frequencies of (A) HCDR1, 2, 3 (B) LCDR1, 2, 3. Comparison of complementarity determining region amino acid frequencies of natural human and mouse antibody repertoire, phage and non-phage antibodies [Color figure can be viewed at wileyonlinelibrary.com]

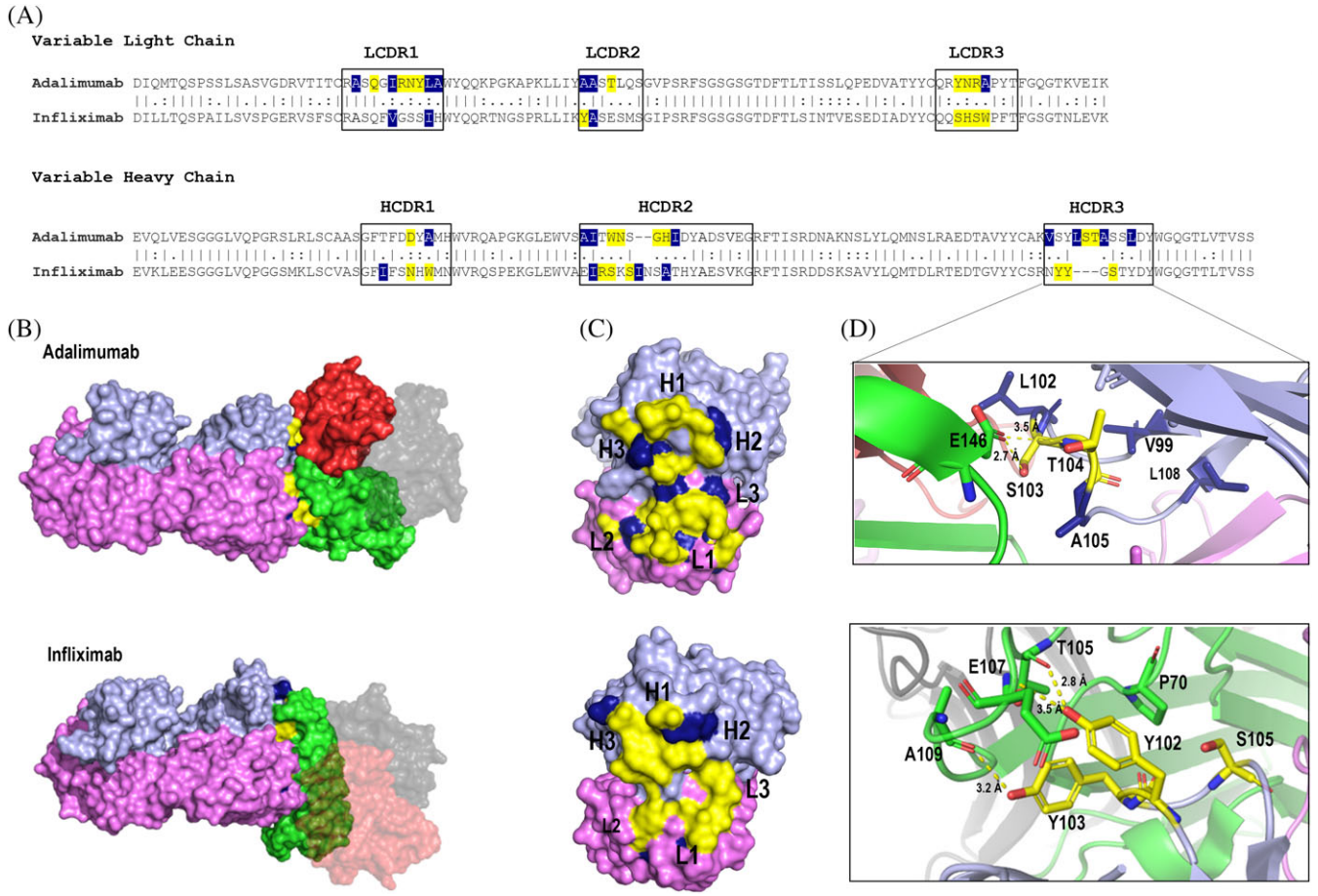



**FIGURE 4** Case study. (A) Alignment of variable heavy and light chain amino acid sequences of adalimumab and infliximab. Complementarity determining regions are labeled with boxes, and interfacing residues and aliphatic residues are highlighted yellow and dark blue, respectively. (B) Top: crystal structure of adalimumab bound to tumor necrosis factor alpha (TNF-α) dimer, bottom: crystal structure of infliximab bound to TNF-α monomer. Light blue: heavy chain, magenta: light chain, red/green/gray: monomers of TNF-α trimer (transparent molecules represent TNF-α monomers that are not involved in antibody–antigen interaction). (C) Top: adalimumab surface with antigen interfacing residues highlighted, bottom: infliximab surface with antigen interfacing residues highlighted. Yellow: interfacing residues, dark blue: aliphatic residues. (D) Interface interaction of HCDR3 of adalimumab (top) and infliximab (bottom). Interfacing residues are in stick form. Yellow: interfacing residues, dark blue: aliphatic residues, green: interacting TNF-α monomer

adalimumab (Ser 103, Thr 104), whereas there were no aliphatic residues in HCDR3 of infliximab (Figure 4A,C). It is important to note that although aliphatic residues form weak non-covalent interactions with multiple residues of TNFα were not represented in Tables 4 and 5 due to their hydrophobic natures. Although weak intermolecular forces such as hydrophobic interactions are key players in antigen binding especially when it is cumulative, their particular roles in affinity and specificity are still to be explored. There are some suggestions that hydrophobic interactions drive protein-protein interactions and H-bonds and salt bridges provide specificity.[57]

Exposed aliphatic residues are distributed all around binding interface of adalimumab, whereas infliximab has limited aliphatic residues (Figure 4C). Although the presence of aliphatic residues exposed on CDR surface might drive antibody-antigen recognition, it is not an advantageous aspect for solubility. Hydrophobic amino acids tend to interact with each other; therefore, self-interaction ultimately leading to aggregation might happen. Our previous result of phage derived antibodies, having more self-interaction characteristics, supports this structural feature. Because specificity is mostly determined by complementarity, aromatic residues with spatial geometries, H-bond donor/acceptor groups, and salt bridges, these features expected to be the main players of specificity. Aliphatic amino acids without any of those stated properties might not contribute much to antibody specificity. Our finding of phage antibodies showing more polyreactivity might also stem from this aliphatic enrichment in CDR regions, especially in HCDR3.

Total charge of HCDR1s of adalimumab and infliximab is −2 and 0, respectively. This is in accordance with our finding of phage derived antibodies having more negatively charged HCDR1s. In one study, they found that negative charge of CDRs, especially HCDR1, is more important than hydrophobicity or beta-sheet propensity for aggregation.[34] In addition to aliphatic content, charge driven molecular forces might contribute to undesired biophysical properties of phage display derived antibodies.

**TABLE 4** List of interactions between tumor necrosis factor alpha (TNFα) and adalimumab

| TNFα | | Adalimumab | | |
|---|---|---|---|---|
| Residue | BSA[a] (%) | Residue | CDR | Interaction |
| ASN 137[b] | 77 | ASP 31 | H1 | Interface |
| ILE 136[b] | 44 | TRP 53 | H2 | Interface |
| VAL 91[b] | 15 | GLY 56 | H2 | Interface |
| THR 79[b] | 82 | ASN 54 | H2 | H-bond |
| ASN 92[b] | 56 | ASN 54, GLY 56 | H2 | H-bond |
| SER 81[b] | 100 | ASN 54 | H2 | H-bond |
| GLU 135[b] | 33 | ASN 54 | H2 | H-bond |
| LYS 90[b] | 50 | ASN 54 | H2 | H-bond |
| SER 147 | 36 | HIS 57 | H2 | H-bond |
| ALA 145 | 55 | HIS 57 | H2 | H-bond |
| GLU 146 | 69 | SER 103, THR 104 | H3 | H-bond |
| GLU 23 | 44 | GLN 27 | L1 | Interface |
| GLY 66 | 92 | TYR 32 | L1 | Interface |
| ASP 140 | 30 | ARG 30 | L1 | H-bond |
| LYS 65 | 78 | ARG 30, ASN 92 | L1, L3 | H-bond |
| GLN 67 | 86 | ASN 31, TYR91 | L1, L3 | H-bond |
| ALA 111 | 76 | THR 53 | L2 | H-bond |
| PRO 20 | 34 | ARG 93 | L3 | H-bond |
| GLU 23 | 44 | ARG 93 | L3 | H-bond, salt bridge |

BSA = buried surface area; CDR = complementarity determining region.
[a]Buried surface area % = buried surface area/accessible surface area × 100.
[b]Other TNFα monomer of natural trimer.

**TABLE 5** List of interactions between tumor necrosis factor alpha (TNFα) and infliximab

| TNFα | | Infliximab | | |
|---|---|---|---|---|
| Residue | BSA[a] (%) | Residue | CDR | Interaction |
| GLU 110 | 32 | ASN 31 | H1 | Interface |
| TYR 141 | 76 | TRP 33, ARG 52 | H1, H2 | Interface |
| ASP 140 | 58 | ARG 52 | H2 | H-bond, salt bridge |
| GLN 67 | 89 | SER 53, SER 55, ILE 56 | H2 | H-bond |
| THR 105 | 79 | TYR 102 | H3 | H-bond |
| GLU 107 | 42 | TYR 102 | H3 | H-bond, salt bridge |
| ALA 109 | 32 | TYR 103 | H3 | H-bond |
| PRO 70 | 78 | SER 105, TYR 103 | H3 | Interface |
| PRO 70 | 22 | TYR 50 | L2 | Interface |
| SER 71 | 93 | TYR 50 | L2 | Interface |
| HIS 73 | 58 | TYR 50 | L2 | H-bond, salt bridge |
| ARG 138 | 69 | SER 91, HIS 92 | L3 | H-bond |
| ASN 137 | 86 | HIS 92, SER 93, TRP 94 | L3 | H-bond |
| ASP 140 | 42 | TRP 94 | L3 | Interface |

BSA = buried surface area; CDR = complementarity determining region.
[a]Buried surface area % = buried surface area/accessible surface area × 100.

## 4 | DISCUSSION

There are studies showing that antibodies discovered by in vitro display techniques have unfavorable biophysical properties compared to the ones derived from hybridoma.[14,24,58] This is mostly due to the lack of immunological tolerance system as opposed to in vivo systems. Although there are such limitations in in vitro display screening systems, they, especially phage display, are the fastest growing antibody discovery/engineering platform in biopharmaceutical research due to their ease of use and proven successes.[24] It is previously documented that biophysical properties of phage display derived antibodies might not be as good as other antibodies.[37] However, there is no molecular understanding about this phenomenon.[37] Although there are some sequence characteristics documented responsible for deteriorated biophysical properties of antibodies,[34,37,49,59] there is no known study about physicochemical analysis of phage display derived antibodies. We argued that physicochemical differences in CDR regions of phage display derived antibodies should play a major role in their undesired biophysical characteristics. First, we found that phage display derived therapeutic antibodies show more self-interaction (or aggregation) and polyreactivity based on their experimental biophysical assay results (Table 2). To understand molecular determinants leading to these disadvantageous properties, physicochemical properties of CDR regions of 137 different therapeutic antibodies were analyzed. Multiple significant relationships were found in physicochemical characteristics of both HCDR and LCDR regions. Most profoundly, there is a higher aliphatic content of HCDR3, HCDR2, and LCDR3 for phage display derived antibodies (Figure 2A).

Hydrophobic amino acids are known to contribute to antibody-antigen binding interactions.[47,60] However, aggregation and nonspecific interactions are also correlated with hydrophobic content of CDRs. A trade-off among affinity, specificity, and aggregation should be considered for exposed hydrophobic amino acids in CDR regions. Hydrophobic amino acids can be divided into two classes: aromatic and aliphatic. It is known that there is a difference between antibody paratopes and general protein-protein interaction surfaces, and the most obvious differences are having less aliphatic content and more aromatic residues in paratopes.[61] It is already known that aromatic amino acids (Y, F, W) play essential roles in affinity and specificity due to their π interaction (π-π stacking, cation-π, and other π systems) abilities and spatial geometries needed for antigen complementarity.[47] In one study, a very hydrophobic aromatic motif, "FHW," on HCDR3 was mutated to an aliphatic "AAA" motif to increase solubility and prevent aggregation. Although these mutations increased solubility 13-fold, antibody completely lost its affinity to its target.[60] Aromatic residues are usually enriched in antibody paratopes due to their important contributions to antigen binding. However, aliphatic amino acids, which are smaller and have less hydrophobicity compared to aromatic ones, might have undesired roles in

specificity and solubility. Our findings about aliphatic enrichment in HCDR3, HCDR2, and LCDR3 might explain higher self-interaction (aggregation) and polyspecificity of phage display derived antibodies compared to non-phage ones. We did not see any significant relationship between aromatic content of CDRs and phage/non-phage class except HCDR2 (Figure 2A). Tyrosine content of phage display derived antibodies is decreased in HCDR2. Tyrosine is one the most abundant residue in HCDRs mostly responsible for antibody specificity and affinity.[45,47] Decreased tyrosine content in HCDR2 might also lead to polyspecificity.

HCDR3 is usually the main region important for polyreactivity. When HCDR3 of a polyreactive antibody is replaced by HCDR3 of a monoreactive antibody, polyreactivity was completely lost.[62] This confirms the importance of HCDR3 in specificity. Another study pointed out that flexible CDR regions make antibodies more polyreactive,[63] and it is known that large residues reduce flexibility.[64] Aromatic hydrophobic amino acids should have a more positive contribution to specificity, whereas smaller and more flexible aliphatic hydrophobic residues might cause polyspecificity. We showed that aliphatic content, especially leucine and isoleucine, is much increased in HCDR3 and HCDR2 of phage antibodies, respectively (Figures 2A and 3A), this might explain non-specificity issues of phage derived antibodies. We also show that glycine is decreased in HCDR3 of phage derived antibodies (Figure 2A). HCDR3 has naturally higher propensity for glycine which is known to contribute antigen binding interfaces.[35,45] Increasing glycine content of HCDR3 region might lead to better binding antibodies.

Aliphatic content of HCDR3, HCDR2, and LCDR3 was increased in phage derived antibodies (Figure 2A). Exposed hydrophobic residues are known to contribute protein aggregation.[65] Aliphatic residues are not as critical as aromatic hydrophobic amino acids in antigen binding; therefore, exposed aliphatic content in HCDR3 and HCDR2 might cause self-interaction leading to aggregation. Another interesting point is that, negatively charged amino acids might contribute to aggregation of antibodies based on their location among CDRs. In one study, it is found that negative charge of CDRs, especially HCDR1, is more important than hydrophobicity or β-sheet propensity for aggregation.[34] Engineering of HCDR1 can be conducted to improve solubility of antibodies. Decreasing negative charge of HCDR1 might be performed to obtain less aggregation-prone variants. As it is shown in Figure 2A, total charge of HCDR1 is more negative in phage display derived antibodies which is also true for our case study (Figure 4). This might also explain our findings about higher self-interaction of phage display derived antibodies.

Although most of significant differences are usually highlighted in HCDR regions, we also showed some significant relationships in LCDR regions, especially LCDR3 which has the highest critical importance.[50] The variable domains of antibodies are made up of variable (V) gene, the diversity (D) gene, and the joining (J) gene. Although $V_H$ domains are encoded by V, D, and J, $V_L$ domains are encoded by V and J genes.[66,67] Natural lack of variable genes may explain low attention given to light chains. However, in one study, it was shown that physicochemical properties of kappa and lambda light chain CDR residues are important for different roles in the humoral immune response[68] and pairing with particular $V_H$ domains.[50] Also, due to their lower tendency of aggregation, $V_L$s are gaining importance as promising candidates for therapeutic applications.[49,50,69] We showed that there is significant increase in aliphatic contents of LCDR3 in phage display derived antibodies (Figure 2B). An increase in aliphatic content of LCDR3 might trigger higher propensity for aggregation. Thus, LCDR regions, especially LCDR3, should also be considered carefully while designing a synthetic phage display library.

All antibodies used in this study are in either approved or in clinical phases. If antibody candidates in such late development stages fail due to biophysical and/or specificity issues, it would be loss of vast amount of money, time and resources. Therefore, it is important to screen, eliminate/improve antibody candidates in earlier stages. Display technologies based on synthetic libraries are the most commonly used techniques for antibody engineering.[46] This study sets important rules for design of the synthetic libraries as well as shedding light into the unwanted properties of clinical antibodies and how to rule them out long before entering into advance development stages. In addition to many physicochemical properties of CDR regions documented here, aliphatic content of HCDR3 should be considered carefully before finalizing library design. Decreasing frequency of aliphatic residues in HCDR3 libraries might yield into more specific and less aggregation-prone antibodies, which would have a higher chance of succession in later development stages.

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