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Simplified monomeric VHH-Fc antibodies provide new opportunities for passive immunization

--Manuscript Draft--

Short Title:	Production of monomeric VHH-Fc antibodies
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Author Comments:	

Simplified monomeric VHH-Fc antibodies provide new opportunities for passive immunization

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Abstract

Simplified monomeric monoclonal antibodies consisting of a single-domain VHH, derived from camelid heavy-chain only antibodies, fused with the Fc domain of either IgG (VHH-IgG) or IgA (VHH-IgA) antibodies, are promising therapeutic proteins. These simplified single-gene encoded antibodies are much easier to manufacture and for bulk applications can be produced in plants and in yeast. These merits enabling novel passive immunization applications, such as in-feed oral delivery of VHH-IgA, which successfully protected against a gastrointestinal infection in piglet model.

Introduction

For over a century, passive immunization is known as the answer to infections when no drugs or vaccines are available. In this procedure, convalescent immune sera from individuals recovered from infections are transfused into infected persons to provide immediate protection. Also, serum therapy with antitoxins, *i.e.* antibodies able to neutralize a specific toxin, and toxoids, *i.e.* an inactivated toxin with maintained immunogenic properties, produced by actively immunized horses was used for a long time to treat particular diseases such as diphtheria and tetanus, and also to neutralize venom toxins after snake bites [1]. The advent of recombinant DNA techniques has enabled the precise engineering and production of monoclonal antibodies with higher efficiency and specificity, which also results in less adverse effects. Production of these conventional mammalian monomeric Y-shaped IgGs (Figure 1a) for intravenous application is expensive, while that of dimeric secretory IgAs (SIgAs), which may be applied topically at mucosal surfaces, is highly challenging (Figure 1b).

To produce an IgG, the transgenic cells should carry the genes encoding the heavy and light chains, both of which should be expressed to yield high quantities of assembled active IgG antibodies. The production of SIgAs is even more complex, because they require four different genes. Next to the genes coding for the heavy and light IgA chains in the monomeric IgA (Figure 1c), the genes encoding the J chain and the secretory component should also be present, such that two monomeric IgAs can be assembled by the J chain and secretory component into a single active SIgA (Figure 1b).

How to simplify the production of IgG and SIgA antibodies?

One way to simplify the production of IgGs and SIgAs is to reduce the number of genes required. This can be achieved by connecting the variable domain of the light chain and the heavy chain via a flexible linker in a single reading frame, which is called a single-chain variable fragment (scFv). Although scFv and scFv-fusion antibody drugs are available on the market or under clinical investigation [2,3], designing of the linker is crucial in obtaining active scFvs, which remains tedious and an empirical process. The success depends on the precise stoichiometric pairing of the variable domains, which is governed by the flexibility and length of the empirically designed linker. An effective alternative is to replace the antigen-binding Fab fragment by a single-domain VHH or nanobody derived from heavy-chain only antibodies (Figure 1d) found in camelids [4]. The VHHs are more compact and small (~15 kDa) with a stable immunoglobulin fold, making them more robust than scFvs [5]. Furthermore, the structural properties of VHHs most often enable the targeting of epitopes inaccessible to conventional antibodies (e.g. deep enzyme clefts), which broadens the range for finding neutralizing epitopes. Also, because all epitope binding information is conferred to a stable single domain, fusing these VHHs genetically to other proteins is much easier than scFvs. For instance, the VHHs can be fused to a fluorescent protein such as GFP, making them interesting new tools to study and visualize the VHH targets in *in vivo* intracellular localization studies [6].

To obtain simplified VHH-Fc antibodies, the VHH of interest is grafted on the Fc domain of an IgG (VHH-IgG) or and IgA (VHH-IgA) at the hinge region [7] (Figure 2b). This results in bivalent monomeric VHH-Fc fusions that can easily be produced in different platforms, as they are encoded by only a single gene and have been demonstrated to express at high levels in plant seeds (e.g. *Arabidopsis* and soybean) and yeast (*Pichia pastoris*). Moreover, the VHH-Fc fusions gain the particular properties of the Fc region, such as increased half-life and effector functions.

To obtain simplified SIgA antibodies (sSIgAs), the J chain and secretory component have to be co-expressed with the VHH-IgA molecule [7-9]. Several methods could be used for introducing the three genes in the same cell, such as simultaneous introduction - as a tandem construct or triple co-transformation, or rather successive introduction - either by successive super-transformation or genetic crossing. In dicot seeds, the highest level of sSIgA expression has been obtained by first identifying transformants expressing high amounts of both the J chain and the secretory component, and super-transforming or crossing them with plants expressing the bivalent monomeric VHH-IgA molecule [8]. Indeed, expression of several genes in the same plant cell, even if the same promoter is used, does not necessarily result in the same amounts of transcripts or co-translated constituent protein chains of the sSIgA. Such discrepancy in proportions often results in heterogenous mix, i.e. along with the fully assembled sSIgAs, partially covalently linked assemblies such as bivalent monomeric VHH-IgAs (Figure 2a) without J chain or secretory component (Figure 2d), or tetravalent VHH-IgAs with J chain and without secretory component (dimeric VHH-IgA, Figure 2c), possibly together with free J chain and free secretory component [7]. Future efforts in improving production yield will have to enable a higher ratio of assembled sSIgAs over other undesired partial assemblies [7]. However, it should be noted that even partially assembled sSIgAs in the heterogenous mixture can be efficacious [7,10]. Hence, for most mucosal applications e.g. enteric antibacterial antibodies in gut lumen, a heterogenous mix of VHH-IgA-based antibody formats may not be a drawback [7].

Applications of VHH-IgG antibody fusions

Quite some VHH-IgGs are being explored for new applications and characteristics. For passive immunization against bacteria and viruses, VHHs and derived fusions are being tested for their neutralization efficacy. A recent example highlights the innovative potential of modular VHH fusions, where four different VHHs, each recognizing a different epitope of diverged influenza hemagglutinin molecules, resulting in a tetramer that binds and neutralizes a broad range of influenza A and B viruses. Fusing this VHH tetramer with the Fc of human IgG1 (multidomain VHH-IgG, schematically represented in Figure 2e) resulted in a strongly increased potency and breadth of influenza neutralization [11••].

Also, in cancer, VHH-IgGs are further explored. A VHH binding to nucleolin and fused to the Fc of IgG1 showed increased binding, antiproliferative effects and cytotoxicity against cancer cells compared to the corresponding VHH by itself [12•].

Further, progress is promised in the field of toxin neutralization. While VHHs binding *Clostridium difficile* toxin B with high affinity could not neutralize the toxin, VHH-IgG fusions reached toxin neutralization comparable to the FDA-approved anti-toxin B monoclonal antibody [13]. Similarly, fusion of VHHs binding botulinum neurotoxin A with an Fc of IgG1 significantly increased the circulation time in the blood and provided a much higher efficacy than the VHHs they were derived from [14].

The use of VHH-IgGs has also been evaluated to prolong the passive immunization and protection of newborn piglets provided by the antibodies in the colostrum and mother milk against F4-producing enterotoxigenic *Escherichia coli* (F4-ETEC). By fusing VHHs binding the subunit of the F4 fimbriae of the pathogenic bacteria to the Fc of porcine IgG antibodies, monomeric F4-specific VHH-IgGs were obtained [7]. Adding these VHH-IgG antibodies to F4-ETEC bacteria resulted in aggregation and large clumps of the F4-carrying bacteria, and also inhibited the attachment of the F4-ETEC to gut villous enterocytes *in vitro* [7]. It was thus predicted that these VHH-IgG antibodies would also prevent colonization of F4-ETEC in the gut, but surprisingly oral consumption of these VHH-IgGs did not, whereas the corresponding F4-specific SIgAs did protect against an F4-ETEC challenge, demonstrating that the choice of Fc fragment is crucial in its efficacy [7] (see below).

Applications of SIgAs and derived VHH-IgA antibody fusions

There are less examples of SIgA-based approaches for passive immunization, although this seems the way to go for pathogen neutralization at the mucosal surfaces. The main reason for this is the difficulty in manufacturing of SIgA antibodies. However, several experiments showing SIgA-mediated passive immunization warrant further investigation. For example, specific SIgA antibodies were shown to enchain growing *Salmonella* bacteria in cloned clusters and to prevent conjugation of drug resistance-carrying plasmids among different strains [15••]. Also, piglets fed with the simplified anti-F4 sSIgA antibodies were protected against F4-ETEC colonization after a challenge with F4-ETEC [7]. Furthermore, Virdi *et al*. recently showed that the single-gene encoded monomeric anti-F4 VHH-IgAs are also efficacious in protecting piglets against F4-ETEC challenge, not requiring the assembled sSIgA format [16••]. Why these monomeric VHH-IgAs did provide *in vivo* protection, in contrast to the monomeric VHH-IgGs, remains an open question. Better stability and higher retention at the gut mucosa may be the plausible reasons for the edible VHH-IgA's efficacy.

How general is the VHH-IgA antibody approach?

The single-gene encoded monomeric VHH-IgA format has been proven to be efficient for gut mucosal protection in the porcine model [7,16••]. Being a highly related model for the human

gut, this is likely to encourage and boost research for prophylactic and therapeutic use of this type of VHH-IgA antibodies. Such future body of research against a variety of mucosal targets will unravel how general the VHH-IgA approach can be. Below are the latest applications exploring VHH-IgA-based approaches.

Enterohemorrhagic *E. coli* (EHEC) is a foodborne pathogen that colonizes the bovine gastrointestinal tract. Saberianfar *et al*. describe the isolation of a VHH specific for the intimin adhesin that is involved in the colonization and its fusion with the Fc domain of IgA [17•]. Transgenic *Nicotiana benthamiana* plants carrying this VHH-IgA, the J chain and the secretory component, produced and assembled secretory VHH-IgA antibodies in their leaves, of which one (VHH10-IgA) bound to the most prevalent EHEC serotypes and neutralized the adhesion to epithelial cells *in vitro* [17•].

Another recent publication describes the fusion of VHHs directed against the *Campylobacter* flagella and major outer membrane proteins, to the constant domain of chicken IgA [18•]. *Campylobacter jejuni* causes a zoonotic infectious disease in humans, leading to major health problems; chickens are considered as the main reservoir. The designer chimeric antibodies were effectively produced in *N. benthamiana* leaves and *Arabidopsis thaliana* seeds, and they did not only bind to their purified antigens but also to *Campylobacter* bacterial cells. In addition, the anti-flagellin chimeric antibodies reduced the motility of *Campylobacter* bacteria *in vitro*.

Both studies showed a strong effect of the VHH-IgAs *in vitro*, but it is now crucial to also test the effect of the anti-EHEC and anti-*Campylobacter* VHH-IgAs *in vivo* in the target host.

Plant seeds and yeast as scalable production platforms for VHH-Fc antibodies

Many VHH-Fc applications are possible, and the results of some case studies are promising. In this respect, two different systems should be considered: on the one hand the parenteral administration of antibodies and on the other hand the topical, mucosal and diagnostic use of antibodies. Currently, the most frequent use of antibodies is the parenteral application of conventional IgGs administered as injectables. These therapeutic IgGs need to be highly purified and the quality strictly controlled and regulated. Also, the post-translational modifications, of which especially glycosylation pattern, is very important to be characterized to minimize adverse reactions. Therefore, it is logical that IgG monoclonal antibody production is largely confined to mammalian cell expression systems, which are well characterized and deregulated. In this respect, alternative production platforms such as yeast and plant systems are not favored at this time because of their different glycoforms. However, glycoengineering of the yeast and plant cell systems might make these platforms competitive in the future, over mammalian cell culture-based secreted antibodies that require big investments and rely on expensive media and production processes [19]. The high cost of the mammalian production platforms may drive the production of a range of new VHH-IgG based injectables towards well controlled plant and yeast systems.

However, the situation is completely different when the antibodies are used for mucosal or topical administration or for the use in diagnostics for specific infections or allergens. Then different glycoforms are not an issue, and more scalable and cost-effective platform exploration is an important asset. Especially for oral passive immunization, which would require milligram to gram quantities of antibodies per treated individual over days to few weeks. This is much higher than in case of indications addressed by intravenous antibodies that typically have higher circulatory half-life of a several days in the blood. Fulfilling this high demand in a cost-effective way with current mammalian cell-based manufacturing would be a big challenge. Moreover,

for mucosal passive immunization the production of SIgAs in mammalian cells gives very low yields and is therefore not feasible. Thus, for many new applications scalable production platforms will be needed, where plant and yeast expression systems are getting due attention [20,21].

Here, we specifically focus on the use of the plant seeds and on *P. pastoris* cultures as production platforms, because the simplified VHH-IgG and VHH-IgA antibodies produced in these systems do not need to be purified for oral applications; moreover, they are scalable and cheap. In addition, unlike mammalian expression systems, they do not harbor the potential risk of human-specific virus contamination [22].

De Meyer *et al*. compared the production of several VHH-IgG fusions in stably transformed *Arabidopsis* seeds, in transiently expressing *N. benthamiana* leaves and in *P. pastoris* cultures [23]. Importantly, the VHH-IgGs were equally and successfully produced as bivalent monomeric functional entities in the three platforms. The main advantage of the transient expression in *N. benthamiana* and production in *P. pastoris* cultures is the speed by which the antibodies can be obtained, and thus these are interesting platforms in an initial stage of experimentation [24]. However, the plant seed platform has the advantage that once the seed stocks are established, they can be stored at ambient temperature for years while the resident antibodies remain stable and functional [16••]. Seeds are also beneficial platform when large amounts of antibodies are needed for oral applications [25]. Moreover, seeds allow to uncouple the production phase and purification phase. Thus, only when the antibodies are needed, for instance to treat an infectious outbreak, they would have to be extracted from the stockpiled seed stocks.

Expression levels of VHH-IgGs in seeds of the model plant *Arabidopsis* were up till 1% of seed weight and several of the sSIgAs accumulated to about 0.2% of seed weight, of which 50% was assembled with the J chain and secretory component [7,8,16••]. Accumulation of several simplified VHH-IgA antibodies in soybean seeds was found to be 0.2% of the seed weight, as high as in *Arabidopsis* seeds [8,16••]. Moreover, and importantly, the soybean-produced unpurified monomeric VHH-IgAs were as protective in an *in vivo* challenge experiment as the *Arabidopsis* seed-produced antibodies [16••]. These results allude to the potential of producing tons of soybeans containing kilograms of therapeutic antibodies for oral treatment of gastrointestinal infections not only in animals but also in humans.

Also, tobacco seeds are very efficient in assembling functional antibodies and they are amenable to upscaling [26]. Tobacco seeds, unlike tobacco leaves, do not contain high levels of alkaloids. Additionally, since tobacco is not a food crop it is highly unlikely to accidently contaminate the food chain.

Last but not least, we want to highlight the use of *P. pastoris* as alternative for the production of cost-effective large amounts of VHH-Fc antibodies. The recent finding that the medium of *P. pastoris* cultures secreting monomeric VHH-IgAs could be spray-dried (a cheap processing technique of liquids with active components) without losing functionality, is a breakthrough for VHH-Fc product valorization. Not only can the spray-dried powder be stored as such, but it can also be admixed in the feed or food when needed. This is expected to open many new opportunities and applications.

Concluding remarks

Formatting VHHs by fusing them to the Fc domain of conventional IgG or IgA antibodies has become a commonly applied approach nowadays. These fusions can be used for passive

immunization against infections and toxin neutralization, and also to prevent a gastrointestinal infection upon oral administration. These single gene encoded monomeric antibodies, easily and cost effectively produced in the scalable platforms such as yeast and soybean seeds, opens new avenues to prevent or treat bacterial and viral infections at mucosal surfaces. Glycosylation is an important issue to consider for many antibodies based injectable passive immunization applications, but in several others like oral and topical route heterologous glycans may be of less concern, especially in such routes exploring the scalable manufacturing capabilities of plant and yeast expression system is a strong benefit.

Conflict of interest statement

Nothing declared.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest

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FIGURES

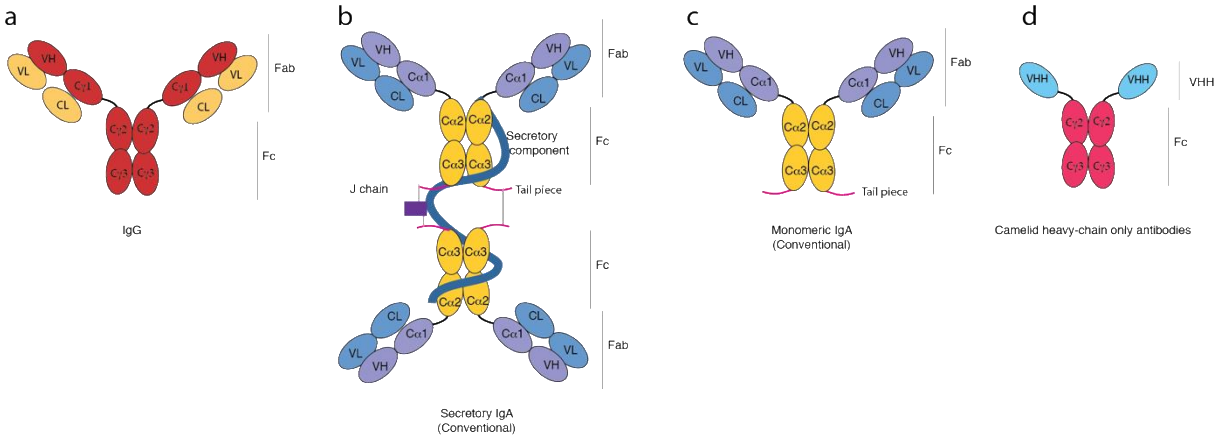

Figure 1. Conventional IgG and IgA antibodies, and the Camelidae heavy-chain only antibodies.
(a) Conventional mammalian monomeric IgG antibodies are Y-shaped molecules that consist of two identical heavy and two identical light chains connected by disulfide bonds. The arms of the Y form the Fab (Fragment, antigen-binding) domain, which binds to the antigen and is composed of one constant and one variable domain from each heavy and light chain. The base of the Y is called Fc (Fragment, crystallizable) and is composed of two constant domains from the heavy chains. (b-c) Mammalian secretory IgA antibodies (b) are a dimer consisting of two Y-shaped IgA monomers (c) covalently joined by the J chain (purple rectangle) via disulfide bonds (black lines) and wrapped by the secretory component. (d) In camelids, next to conventional IgG antibodies, also heavy-chain only IgG antibodies are produced. VL: variable domain of light chain; CL: constant domain of light chain; VH: variable domain of heavy chain; VHH: single-domain antigen-binding domain; Cα1, Cα2, Cα3: constant domains of alpha heavy chain (IgA); Cγ1, Cγ2, Cγ3 constant domains of gamma heavy chain (IgG).

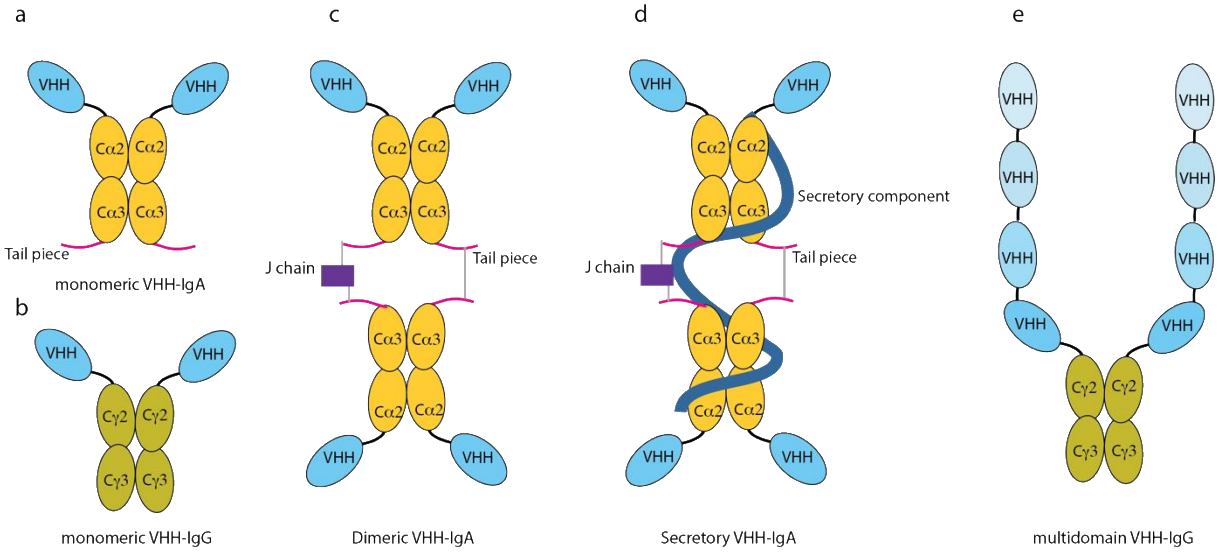

Figure 2. Schematic representation of the simplified VHH-Fc fusion antibodies.
(a-b) Monomeric VHH-Fc fusions consisting of VHHs grafted on to the flexible hinge of the Fc domain derived from either IgA (VHH-IgA, a) or IgG (VHH-IgG, b). (c) Two monomeric VHH-IgAs bind tail-to-tail via incorporation of the J chain (purple rectangle), and form a tetravalent (four VHHs) dimeric IgA. (d) The association of the secretory component (blue band) with the dimeric IgA results in the formation of a secretory VHH-IgA. (e) Four VHHs

specific for different epitopes can be fused together via a flexible linker and grafted on to a Fc domain of IgG to form a multispecific/multidomain VHH-IgG. VHH: single-domain antigen-binding domain of camelid heavy-chain only antibody; Cα2, Cα3, Cγ2, Cγ3: constant domains of heavy chain.

Recombinant simplified monomeric VHH-Fc antibodies provide new opportunities for passive immunization

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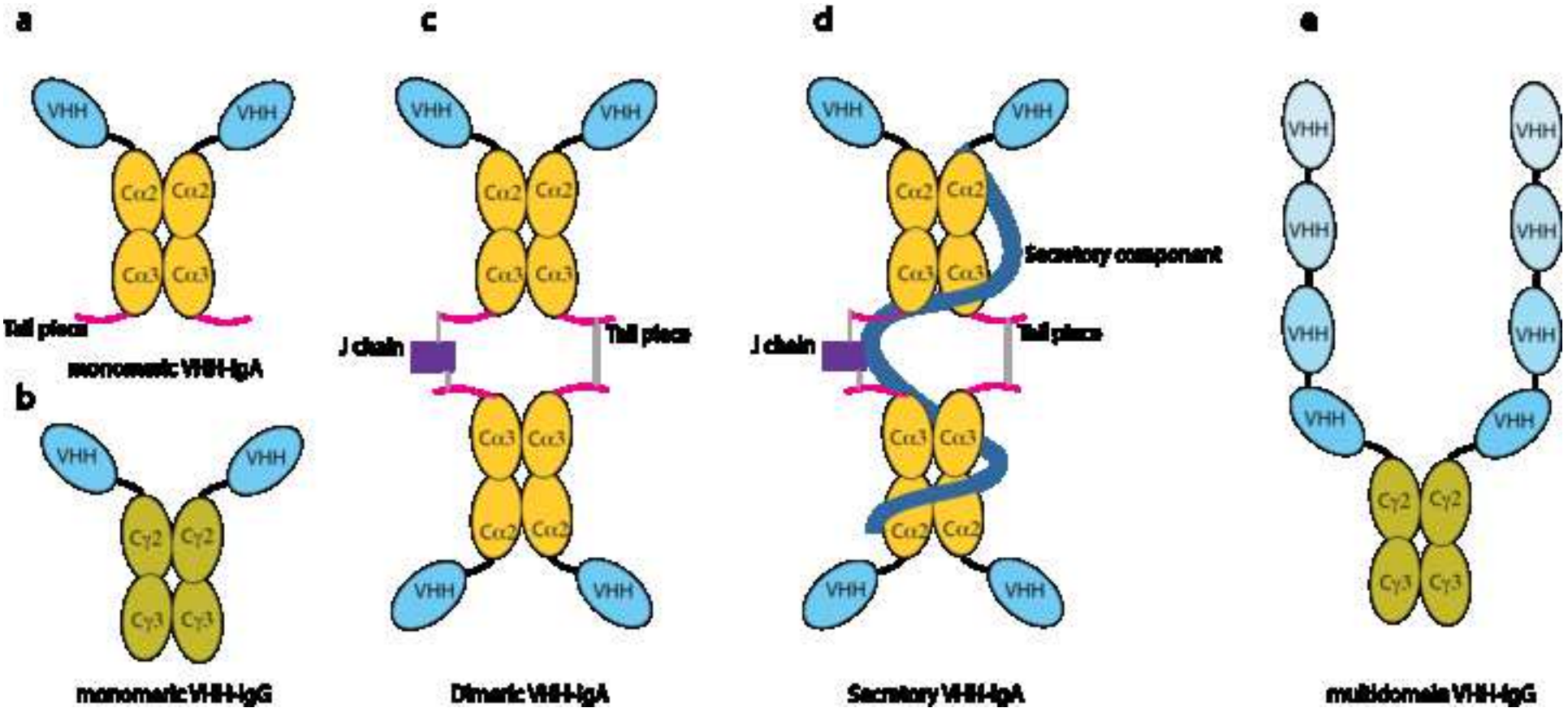
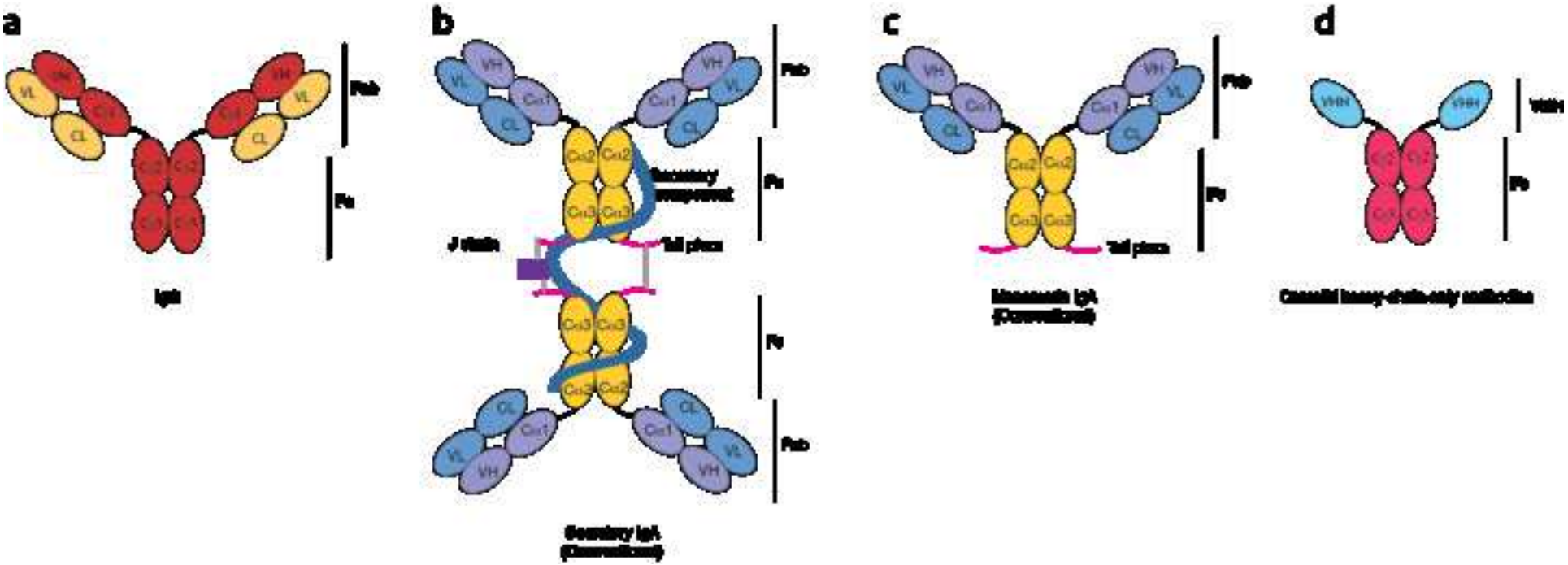
Conflict of interest statement

Nothing declared.





31st October 2019

Rebuttal to the reviewer comments

We would like to take the opportunity to thank the reviewers for their insightful and constructive comments. We have revised the manuscript guided by these comments and believe this further improves the article. We have highlighted the key changed in red font.

Thank you.

Ann Depicker
Henri De Greve
Vikram Virdi
Shruti Bakshi

Our point-by-point reply to each comment is as below.

Reviewer comments:

Reviewer 1

I read with interest the manuscript by De Greve, et al. I found it to be informative and concise and as such appropriate for publication. I recommend accepting the manuscript after minor revisions as follows:

1. The manuscript will benefit from English language editing. In general use of language is fine; however, there are a number of grammatical and other linguistic errors that need to be corrected.

Reply: We thank the reviewer for the appreciation of the manuscript. As requested by the reviewer, we have corrected the grammatical and linguistic errors in the revised manuscript.

2. It would have been helpful to have page and/or line numbers, as this would have facilitated the review process.

Reply: Thank you for this remark. We have added the line numbers now.

3. Abstract: "..: IgG or IgA antibodies are new promising therapeutic proteins". Are they really new? When was the first example in the clinic?

Reply: To clarify this further, we have modified the abstract as follows- (Line 15-20)

"Simplified monomeric monoclonal antibodies consisting of a single-domain VHH, derived from camelid heavy-chain only antibodies, fused with the Fc domain of either IgG (VHH-IgG) or IgA (VHH-IgA) antibodies, are promising therapeutic proteins. These simplified single-gene encoded antibodies are much easier to manufacture and for bulk applications can be produced in plants and in yeast. These merits enabling novel passive immunization applications, such as in-feed oral delivery of VHH-IgA, which successfully protected against a gastrointestinal infection in piglet model."

4. A number of terms may not be familiar to non-experts, e.g. antitoxins, toxoids, etc.

Reply: Thank you for this remark. We have clarified these terms in the revised manuscript. (Line 25)

"Also, serum therapy with antitoxins, *i.e.* antibodies able to neutralize a specific toxin, and toxoids, *i.e.* an inactivated toxin with maintained immunogenicity properties, produced by actively immunized horses ……"

5. Page 1. Last paragraph. Is it really the case that the heavy and light chains need to be expressed at the same levels, etc., etc? I am aware of many examples where efficacy is not influenced by unequal production of the two chains. A number of examples in the literature show that excess of one chain does not negatively influence performance.

Reply: We agree with the reviewer. To clarify this further, we have modified the text in the revised manuscript. (Line 35)

" To produce an IgG, the transgenic cells should carry the genes encoding the heavy and light chains, both of which should be expressed to yield high quantities of assembled active IgG antibodies. The production of SIgAs is even more complex, because they require four different genes. Next to the genes coding for the heavy and light IgA chains in the monomeric IgA (Figure 1c), the genes encoding the J chain and the secretory component should also be present, such that two monomeric IgAs can be assembled by the J chain and secretory component into a single active SIgA (Figure 1b). "

6. Page 2. Statement in the penultimate sentence will benefit from a reference.

Reply: We have added the following references and also further clarify the statement as below.
Virdi *et al.* [7], Bakshi *et al.,* 2019 [10] (Line 83)

"Hence, for most mucosal applications e.g. enteric antibacterial antibodies in gut lumen, a heterogenous mix of VHH-IgA-based antibody formats may not be a drawback. "

7. Page 4. "Fulfilling this high demand…with mammalian cell-based… is highly questionable" And yet this is the technology currently used commercially! I agree with the authors but a reference or two perhaps might strengthen this position.

Reply: Thank you for this comment. We realize is crucial to clarify this point and hence have modified the first two paragraphs of this section as follows. (Line 154-182)

Many VHH-Fc applications are possible, and the results of some case studies are promising. In this respect, two different systems should be considered: on the one hand the parenteral administration of antibodies and on the other hand the topical, mucosal and diagnostic use of antibodies. Currently, the most frequent use of antibodies is the parenteral application of conventional IgGs administered as injectables. These therapeutic IgGs need to be highly purified and the quality strictly controlled and regulated. Also, the post-translational modifications, of which especially glycosylation pattern, is very important to be characterized to minimize adverse reactions. Therefore, it is logical that IgG monoclonal antibody production is largely confined to mammalian cell expression systems, which are well characterized and deregulated. In this respect, alternative production platforms such as yeast and plant systems are not

favored at this time because of their different glycoforms. However, glycoengineering of the yeast and plant cell systems might make these platforms competitive in the future, over mammalian cell culture-based secreted antibodies that require big investments and rely on expensive media and production processes [19]. The high cost of the mammalian production platforms may drive the production of a range of new VHH-IgG based injectables towards well controlled plant and yeast systems.

However, the situation is completely different when the antibodies are used for mucosal or topical administration or for the use in diagnostics for specific infections or allergens. Then different glycoforms are not an issue, and more scalable and cost-effective platform exploration is an important asset. Especially for oral passive immunization, which would require milligram to gram quantities of antibodies per treated individual over days to few weeks. This is much higher than in case of indications addressed by intravenous antibodies that typically have higher circulatory half-life of a several days in the blood. Fulfilling this high demand in a cost-effective way with current mammalian cell-based manufacturing would be a big challenge. Moreover, for mucosal passive immunization the production of SIgAs in mammalian cells gives very low yields and is therefore not feasible. Thus, for many new applications scalable production platforms will be needed, where plant and yeast expression systems are getting due attention [20,21].

8. Page 5. Reference [18] is hardly an authoritative reference on the topic, the authors can do much better!

Reply: Thank you for this suggestion, as you will see in point 7, the paragraph has been modified and references [20,21] now replace the previous reference [18]. (Line 180)

9. Page 5. GRAS status. This is only a recommendation by the US FDA. It makes no difference in other jurisdictions, e.g. Europe!

Reply: Thank you for this remark. We have modified the text as follows (Line 184) and added reference [22]

"Here, we specifically focus on the use of the plant seeds and on *P. pastoris* cultures as production platforms, because the simplified VHH-IgG and VHH-IgA antibodies produced in these systems do not need to be purified for oral applications; moreover, they are scalable and cheap. In addition, unlike mammalian expression systems, they do not harbor the potential risk of human-specific virus contamination [22]."

Reviewer 2

In this review an analysis on the potential of VHH antibodies fused to IgG/IgA Fc regions is presented, highlighting cases based on the use of plants and yeast as the expression host. In my opinion the manuscript has a proper structure and described the technology properly.

However, some aspect require attention:

10. The authors should consider to mention -plants and yeast- in the title as these are the hosts in which the review is focused on.

Reply: We thank the reviewer for the positive remarks on the manuscript structure and the description. We acknowledge your advice, but with this article we intent to spotlight the VHH-Fc antibody format, to usher and steer exploration of VHH-based multivalent antibody formats. The simplicity of expressing single gene VHH-Fc would provide convenience in any expression platform (mammalian, plant or yeast). Every platform would have its due merits depending on the application, and when it comes to antibody applications requiring milligram to grams quantity per individual, yeast and plants may be a good alternative to consider.
Acknowledging your comment, we have clarified this not only in the abstract but in entire manuscript. And for brevity we even removed the work 'recombinant' from the title further shortening the character length.

11. What about glycosylation? This aspect is not discussed and is critical in terms of safety (generation of undesired immunogenicity) and for certain applications such as cancer therapy, in which the effector properties of the antibody are important and could be affected by the glycosylation patterns.

Reply: We agree with the reviewer. Your comment brought to our attention that the readers will benefit from clear distinction of the VHH-Fc antibodies that may be injected from the ones that may be orally or topically applied. The heterologous glycosylations are deemed important for preventing adverse reaction when injected, not so when their role is topical at mucosal surface. We clarify these points in lines: Line 155-175 (Also taking into account comment 7 of reviewer 1).

12. The authors must enlist much in detail the advantages of the plant- and yeast-based production platforms, e.g. by providing specific advantages in terms of cost, scalability and biosafety.

Reply: We have discussed the advantages of the plant- and yeast-based production platforms for VHH-IgA production in the revised manuscript (Line 155-175). (please see the text of comment 7 of reviewer 1)

13. Expression approaches could be also mentioned briefly. Moreover, a comparison of such platforms should be provided to contrasts their advantages and limitations. What about the singular glycosylation machinery of yeast compared to plants? What about glycoengineering approaches in these organisms?

Reply: We agree with the reviewer that these are very interesting topics. However due to space limitations of the manuscript (2000 words) addressing the topic is beyond the scope of this review. However, in this revised manuscript we have introduced the readers to how this post-translational modification may play a role in VHH-Fc applications, stating our own opinion when it comes to oral/topical mucosal or injectable route. Additionally, in Line 166 we also added reference of Van Landuyt et al. 2018, which is a more pertinent review on glycosylation and covers the issues in more details.

14. In the concluding remarks section, the authors should provide a real analysis of the current knowledge and provide future directions for the field. what are the challenges in terms of regulatory approval? Are there some challenges related to yields? What happens with the glycosylation aspect in these antibodies? Remains unexplored? Is it critical? In what other diseases these antibodies could be applied? I consider these questions of key relevance to enrich this section.

Reply: Please note there is limit to the concluding remark section. In light of your advice, we have added following text in the concluding remarks section (Line 228-231) and where fit, we have attempted to touch

these points elsewhere in the manuscript. With no regulatory examples of VHH-IgA (or recombinant IgAs) for edible/topical mucosal application the regulatory pathway remains unexplored and at this point of time is broadly hypothetical, hence we have chosen not to elaborate on it.

"Glycosylation is an important issue to consider for many antibodies based injectable passive immunization applications, but in several others like oral and topical route heterologous glycans may be of less concern, especially in such routes exploring the scalable manufacturing capabilities of plant and yeast expression system is a strong benefit."