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Review Article

Novel Bio-Molecular Properties of Nanobodies (Nbs) and Sybodies (Sbs), Links with Predecessor Conventional Antibodies, for Enhanced Applications in Immuno-Therapeutic Drug Development and Precision Medicine

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Abstract

Nanobodies ((Nbs) and sybodies (Sbs) are single domain antibodies (sdAbs) based only on the variable V_HH domain of heavy chain of whole antibodies molecule, with immune capability functions but smaller than conventional antibodies. They were firstly discovered in a *Camelid* species.

The goals of this article are to bring to fore the qualities of nanobodies and sybodies, briefly trace their background, connect with their predecessor monoclonal antibodies (mAbs) in areas of utilities for immunotherapy and medical care, present attributes of sdAbs-cell membrane receptors interactions. and pharmacokinetics observed so far, based on various studies we retrieved.

Nanobodies are small sized, have unique paratope architecture, yet highly stable, possess good level of solubility, now inexpensive to produce, capable of binding on to antigenic or target molecules and their small size enable them and their paratopes to easily reach difficult to access epitopes of target proteins. Sybodies are synthetic forms of nanobodies which can be easily selected under desired defined conditions, possess uniquely small sizes that can be aerosolized for therapeutic administration, have high penetrative-dispersive power.

This is a novel presentation in its own rights and a selfless contribution to the ongoing science of antibody therapeutics and diagnosis. Antibodies such as mAbs have proved their mettle in being successfully developed into clinical immunotherapeutic tools. It is equally important to note that prior to the discovery of sdAbs (nanobodies and sybodies), mAbs which are bigger that sdAbs, were long discovered to meet therapeutic needs in tackling of ailments that challenge man. Nanobodies and sybodies have come in to compliment the mAbs in clinical immunotherapy.

sdAbs (nanobodies and sybodies) have beneficial attributes related to their unique properties which are utilized in diagnosis, structural determination, molecule-delivery tools in the human body, structure and non-structure based drug design and development and enhancing microscopy for clearer content definitions in cell and molecular biology. Engagement of appropriate pharmacokinetic properties and maximized Ab concentrations are important considerations in development of optimized sbAb based immunotherapy in non-conjugated and reconstructed bioengineered formats and in conjugated design forms of these drugs. There is the need for continuous testing of molecules to be conjugates onto sdAbs to develop more sdAb immunotherapeutic conjugates, at optimized combinatorial synergistic potentials of potency, to shore-up our pool of successful therapeutic agents.

Keywords: *Antibodies; Nanobodies; Sybodies; Single Domain; Therapy; Diagnosis; Precision Medicine*

Introduction

Nanobodies and sybodies belong to the class of single domain antibodies (sdAbs) and are bio-molecules with immune capability functions like conventional antibodies, but are characteristically smaller than conventional antibodies.

Nanobodies were first discovered before sybodies and are characteristically small sized antibodies with size range molecular weight 12 - 15 kDa (about $1/10^{th}$ size of antibodies [1-3] characterized by presence of heavy chain (heavy chain antibodies-hcAbs) with single domain antigen binding fragments. Since they lack the light chain of a conventional antibody, nanobodies are reduced to single domain of the heavy chain left [4-7]. The single domain of nanobodies are called V_HH domain antibodies (variable heavy domain of heavy chain antibodies). These features have been depicted on the classical and crystal structures of Nanobody bio-molecules on figure 1a-1c and figure 2a and 2b with the data references shown.

Nanobodies were originally discovered from a Camelid and have since then been found in other Camelids like Ilamas and Appacas and now in nurse sharks [8,9]. The first nanobody based drug is Caplacizumab, approved by the US-FDA in 2018, developed for treatment of acquired thrombotic thrombocytopenic purpura (aTTP) [7,10-12].

Sybodies are synthetic nanobodies [13,14], a product of the benefit of furtherance in developed technology for studying bio-molecules. Like conventional antibodies, nanobodies and sybodies can selectively bind on to specific antigen [14].

Discovery and concept of sdAbs-nanobodies and sybodies

By stroke of chance, some students of an Institution in Brussels came across a strange group of small sized antibodies that did not correspond to anything known bio-molecule in science, in addition to the usual distribution of antibodies which they identified in analyzed samples they were working on in a Laboratory session. Then, two researchers in same institution engaged this uncertainty head-on by characterizing these strange small antibody bio-molecules and discovered that they are actually a new class of antibodies peculiarly characterized by the presence of only the heavy chain to depict a form of a single antigen recognizing domain, thus lacking in the light chains. Based on these unique features, these strange bio-molecules were named heavy chain antibodies (hcAbs). This investigation was performed on a Camelid species [7,9].

As such, the first sdAbs were Nanobodies and were engineered from heavy-chain antibodies (Abs) obtained from a Camelid species [16-18].

At a period in nanobodies' technology, the production of nanobodies from animal species was time consuming, as such technology was further developed that eventually opened up way for the design and production of synthetic forms of nanobodies that were subsequently termed sybodies. sdAbs class of antibodies are showing promise in research applications in biotechnology [19], diagnostics [20-25], tracking treatment progress via imaging [26,27], drug and vaccine developments and drug delivery to tissues [3,4,25,28-30].

A typical sybody is "Designed Ankyrin Repeat Protein" (DARPins). Retrospectively gathered data on this synthetic nanobody revealed that it has a really small size of 12 - 18 kDa, can be rapidly produced in very huge numbers of the magnitude of thousands in *Escherichia coli* bacteria, are active at very low concentrations, highly soluble, all features that have enhanced its capacity to target several disease pathways and be used for targeted delivery of therapeutic drugs and toxins. This has promise for contributing to precision therapeutic medical care [31,32].

Significance and compelling need

Antibodies have proved their mettle in being successfully developed into immunotherapeutic tools to fight diseases like cancer, autoimmune diseases and pathogenic diseases of which includes viruses infections [32-36]. There are already some Food and Drug Admin-

istration (FDA) authorized antibodies (Ab) based drugs, though expensive, can take time to design and develop and their size range may not be easily exploitable for some administration via aerosolized forms that could target most of the respiratory system contained in the human thoracic region. Howbeit, conventional antibodies of the category of mAbs have size range of approximately 150 kDa [3,25,37,38]. This conferred limited tumour and tissue penetrating capacities, coupled to high cost of production [6] and relative to the more recent immuno-molecules of nanobodies and its synthetic variant sybodies, necessitated quest to search for other bio-molecules of the immune mechanism which can pass these limitations to support the successes achieved with use of conventional antibodies for therapy.

The objectives of this study are:

- Highlight novel properties and qualities of nanobodies and sybodies sdAbs.
- Present attributes of these sdAbs cell membrane interactions, and pharmacokinetics based on reviewed past studies.
- Sieve out usage applications of nanobodies and sybodies in supporting immunotherapy and precision medicine.

Links from the use of monoclonal antibodies as predecessors to sdAbs

Monoclonal antibodies (mAbs) furnished us with added options of clinical tool as they emerged as a prevalent treatment in cancer therapeutics and provided a revolutionary breakthrough in cancer therapeutics [25,28,30]. The discovery of nanobodies was by chance, they are smaller versions of antibodies, cleavable out of whole unit antibodies with support from advancements in molecular technology and it's very possible applications for precision medicine. At this period in nanobodies' technology, the production of nanobodies from animal species was time consuming, as such technology was further developed that eventually opened up way for the design and production of synthetic forms of nanobodies that were subsequently termed sybodies; which are bio-molecules that combined features of smaller size, quicker synthesis for application-based designs and use, yet capable of binding onto antigenic sites and target molecules like conventional antibodies and nanobodies.

It is equally important to note that prior to the discovery of sdAbs (nanobodies and sybodies), monoclonal antibodies (mAbs) which are bigger that sdAbs and part of conventional antibodies, were long discovered and rolled into science and met complex therapeutic needs [35,39,40] and supported tackling of diseases that confront man. Records and data shows that the number of approved mAbs has been growing and there are now well over 90 mAbs that have passed the strict clinical tests hurdles and are on approval list of the United States Food and Drug Administration (US-FDA) [13,41] which have been developed for clinical applications against cancer, auto-immune diseases, pathogenic infections among other ailments [33,42,43]. Monoclonal antibodies got selected for their capacity to rattle and distort the normal functions of their target pathogens and deranged cells in cancer ailments. Intact mAbs evoke antibody dependent cell-mediated cytotoxicity (ADCC) and have been engineered to carry molecules targeted for therapy through conjugational devices [15,44,45] and for diagnosis [46-48].

The entry of nanobodies into this foray of immune-based bio-molecules in translational biomedical research and its applications and this sort of clinical usage are now being implemented for sybodies to support tackling of communicable and non-communicable diseases that confront man. Retrospectively retrieved evidence based data, shows that nanobodies and sybodies have come in to compliment the helps which mAbs have offered the therapeutic community to fight diseases [12]. The development of efficient ways to deliver large molecules such as peptides, proteins and nucleic acids across the blood-brain barrier (BBB) has been described as been crucial to future therapeutic strategies for treatment of central nervous system (CNS) disorders [49,50]. This stems from the attribute of small size of antibodies and sdAbs and this could be more robust in delivery to deeper sections of tissues and cells through the use of sdAbs - nanobodies and sybodies which brings in smaller sizes and more intense penetrative power of therapeutic delivery into the body.

Properties of nanobodies beneficial for immuno-therapeutic drug discovery and precision medicine

1. Nanobodies are small sized, yet highly stable. The small size is utilizable to develop aerosolized formulations of nanobodies for immune-therapeutic administration (inhalable therapeutics). They exhibit a rare high level of stability even under adverse conditions of temperature, detergents, glycerol, salt, reducing agents and pH among others [6,7,19,51,52].

2. They are now less expensive to produce, as done using Bacteria and Yeast cells, though it may still be time consuming [19,53].

3. The monomeric single-domain nature of nanobodies form the basis for the flexible engineering of nanobodies, which facilitate conjugation of additional proteins, reporter molecules and drugs [38,54].

4. They are capable of binding on to targets with similar affinity range as conventional antibodies [1,5].

5. They are enabled from their small size and easier flexibility to more readily reach difficult to access epitopes of proteins and target sites, such as the crevices [3,6,51].

6. Nanobodies have become promising clinical therapeutic tools to battle the smallest in size-class of pathogens which infect humans, of which are mostly the viruses, stemming from their high stability and small size, that can "plug and play", albeit "key and lock" on to deeply concealed epitope crevices for neutralization of viral virulence and pathogenic capacity. This is a prospective area of research that also taps into their small-size penetrating power. Wu., *et al*. described them as good fit antibodies for neutralizing viral antigenic peptides and epitopes.

7. Nanobodies can be used as crystallization chaperons for structural investigations of diverse conformational states of flexible membrane proteins [57,58]. This is helping in providing better understanding of the cellular and molecular features of cells which benefit applications processes in therapy and detection of diseases.

8. They are now used to track and manipulate protein function [38].

Properties of sybodies beneficial for immuno-therapeutic drug discovery and precision medicine

1. They can be selected against target proteins within few days, even below 14 days [53,59,60]. This makes them the point-of-call tools for swift pathogen tailored designs, development and clinical trials, dusting strange pathogen triggered epidemic that tend to advance into pandemic proportions. This expands the pool of options to fight such diseases.

2. When it comes to situations that demands generation of binding surfaces that recognize the most lethal bio-molecular conformations of target antigens or peptides, sybodies come in handy as they can be easily selected under such desired defined conditions [38]. This takes advantage of the rapidity of selection, bearing in mind that their specificity in binding and permeability to reach obscurely exposed crevices on target proteins.

3. Like nanobodies, both possessing uniquely small sizes, sybodies can be aerosolized and deployed in inhalable forms of immunotherapy administration [53,60], coupled to their high penetrating power stemming from same uniquely built small sizes and molecular weights.

4. They are highly specific binders and find use to block target proteins of interest for therapeutics [38].

5. Remarkably, there can now be a novel perspective for engaging sybodies in therapeutics by using multiple (greater than two) sybodies which can be carefully designed to coordinately and synergistically bind on to the most implicated epitopes on tar-

get molecules of pathogens and deranged cells of cancer, impede pathogenesis and disease progression or commencement of invasion of human cells by pathogens. This can be a novel corridor to work on and bring it to fruition to support drug action and novel drug development efforts. The synthetic composition and build-up of sybodies, capacity to reach high level affinity, small size and neutralization capacity of sybodies, as shown in its characterizations [13,60], is a plus to engage to get closer and achieve these perspective of immunotherapeutic line of drug design and action, through sdAbs. Walter., *et al*. [53] mentioned the possibility of designing sybody therapy of more than one sybody, such as two or three sybodies.

6. For determining 3D structures of membrane proteins at high resolution by co-crystallization with specific binding proteins like DARPIns sybody, which provide rigid hydrophilic surfaces for stable protein-protein contacts [58,68].

Molecular details of nanobodies and sybodies and mechanism for penetrative capacity to reach epitopes and target areas

Nanobodies mostly posses a specific long extended complementarity determining region (CDR) loop compared to human antibody, which forms a finger-like structure that penetrates into cavities on the antigen surface [61,62]. Even for V_HH lacking the required specific CDR prolate [5,6], the shape of nanobodies also creates a convex paratope that interacts with antigen concave surface in an in-depth manner [16]. Nanobodies are oxidized upon secretion to form disulphide bonds between cysteine pairs despite the absence of a periplasmic intermediate [63]. Some of these features have been depicted on the crystal structure of Nanobody from Ilama Camelid on figure 2, with the data references shown. As such, nanobodies bind specifically to conformational epitopes with picomolar and nanomolar affinity and the crystal structure shown in figure 2 is that of a nanobody NB17 raised specifically against the catalytic core of KDM5B which recognizes methylated lysine residues which is in itself an epigenetic marker from a study conducted by Wiuf., *et al* [58]. Over-expression of this KDM5B has been shown in several studies to cancer cells proliferation and reduces tumour suppression cells - an attribute that conferred novel target for oncological drug discovery on KDM5B [64,65].

Naturally occurring Ankyrin repeat proteins DARPins sybodies consist of Ankyrin repeat modules and mediate protein-protein interactions mainly in Eukaryotic cells [67,70]. Each of these modules has been found to consist of 32 amino acids and folds into a beta-turn followed by two antiparallel alpha helices and a loop that connects to the next repeat. Batchelor., *et al*. [68] from their study observed that Ankyrin repeat modules assemble into elongated and slightly curved structures to put up a concave shaped surface that forms specific contacts with its binding partners. This makes them potent driving tools to carry and deliver drugs and bio-molecules to bind and fit-in at their desired targets, such as diseased cell lines. The study of Stefan., *et al*. [69] revealed that DARPins recognizing the tumour associated antigen EpCAM selected by phage and ribosome display.

The structural build-up of sybodies such as DARPins suits them to track, bind and fit into folded proteins. Unlike nanobodies, Designed Ankyrin Repeat Proteins (DARPins) sybody have been built to lack cysteine residues, implying that they lack disulfide bonds. As such, they can easily fold inside the reducing environments of cellular interiors [38,58,96]. Some of these features have been shown on the 3 Dimensions crystal structure of Sybodies on figure 2a and 2b, with the data references provided alongside [70].

Conventional antibodies are good at detecting and binding denatured or unstructured proteins while DARPins work extremely well on proteins that have folded domains as good fits and binders [63].

Typical forms of nanobodies include monomeric nanobodies [63,104], dimeric nanobodies [104], fusion nanobodies such as: Maltose binding protein-Nanobodies (MBP-Nb) fusion and fusion Nanobodies-hemolysinA (Nb-HlyA) [63,104,113], among others.

Typical forms of sybodies include Affirmers, Adnectins monobodies and DARPins (Designed Ankyrin Repeat Proteins) [38,91] among others.

Typical methods to isolate sdAb nanobodies [3,4,8,9,24,57,62]:

From *camelids* (Camels and Ilamas), cartilaginous fishes (like sharks)

1. Immunize the organism with required antigen.
2. Isolate mRNA coding heavy chain antibody using suitable isolation (or dissociation) technique.
3. Use library construction method based on a cloning sequencing platform like the PCR-extension assembly, coupled to self-litigation (EASeL) to display a large phage Ab library from a naïve non-immunized animal.
4. Screen with phage to identify the clones binding the Ag which are the sdAb nanobodies.

Typical technique for natural production of nanobodies from immune system of humans or mice animal model

- This commences with appropriate immunization of the organism. Then, we split the dimeric variable domains from a common source of antibodies in human or mice immunoglobulin IgG into monomers using appropriate technique. Purification follows to ensure quality of generated nanobodies [18,62].

Other emerging methods for production of other forms of nanobodies (not produced from animal facility):

- Nanobodies can be developed through naïve libraries or construction of a synthetic phage-displayed nanobody library with CDR3 regions randomized by trinucleotides cassettes [25,71-73].
- Bacterial *E. coli* hemolysis system of secretion of nanobodies [63].
- High yield production form *Escherichia coli* periplasm as fusions with maltose binding protein (MBP-Nb fusion) [32].
- Nanobodies can be generated from mRNA/cDNA display *in vitro* before being encoded, tagged and expressed in cells for *in vivo* localization and functional studies of target proteins [2,75].
- From semi-synthetic libraries using Yeast displays [72].
- From Bacterial two hybrids using two plasmids that code for the target of a variety of VHH domains from an immune library and non target proteins [77].
- Fusing V_HH domains to enzyme chloramphenicol acetyltransferase (CAT) enzyme produced in *Escherichia coli*. This screening method alone is not adequate for selection of high affinity nanobodies and as such is used as initial screening to obtain synthetic and naïve libraries [76,77]. Therefore, it narrows the nanobody library pool for easier handling in subsequent more thorough methods of selection.
- Lentiviral screening which commences with inactivated Lentiviral particles that are used to immunize the animal (such Ilama) against a multitude of viral antigens, collection of the Ilama lymphocytes, amplification of the V_HH sequences and its cloning into a lentiviral Plasmid, then use the lentiviral particles that are generated to transfect specific human cells which are then exposed to a lethal dose of the lentivirus. The surviving cells contain V_HH which is stable within cytoplasm and with functional attribute of being capable of neutralizing the virus [55]. This method has been described as inclusive in providing new options for gene therapy via nano-engineering because the selection process for the nanobody here, furnishes information on precise epitopes to be targeted for effective viral neutralization [77].

- From fusion with use of disulphide bond enzyme in which we engage tagging V_HH-GFP fusion with DsbC disulphide bond isomerase to catalyze formation of disulphide bonds in the V_HH domains, leading to production of stable nanobodies [36,79]. Olichon and Surrey who developed this method used camelid derived nanobodies.

Methods for purification of nanobodies

This has been done mostly by affinity chromatography, as demonstrated thus:

- Use of single affinity chromatography performed on supernatant of *E. coli* cultures in which Nanobodies-hemolysinA (Nb-HlyA) fusion system have been secreted [63].
- Use of two affinity chromatography steps on Maltose Binding Protein-Nanobodies (MBP-Nb) fusion form of nanobody [74].
- Affinity chromatography performed on His-tag Protein A of *Staphylococcus aureus* (SpA) affinity resins. This has been developed by Crauwels., *et al* [80].
- Immobilized metal affinity chromatography (IMAC) using Nitrilotriacetic acid (Ni-NTA) beads. This method is reputedly sensitive to proteolytic cleavage with associated undesirable heterogeneity [80].
- Ammonium sulfate precipitation (ASP) and electropositive multimodal chromatography (Capto MMC) for tag-free nanobody integrated purification. A method described as being easy to operate, simple and of low cost [81]. It is a two step purification system.

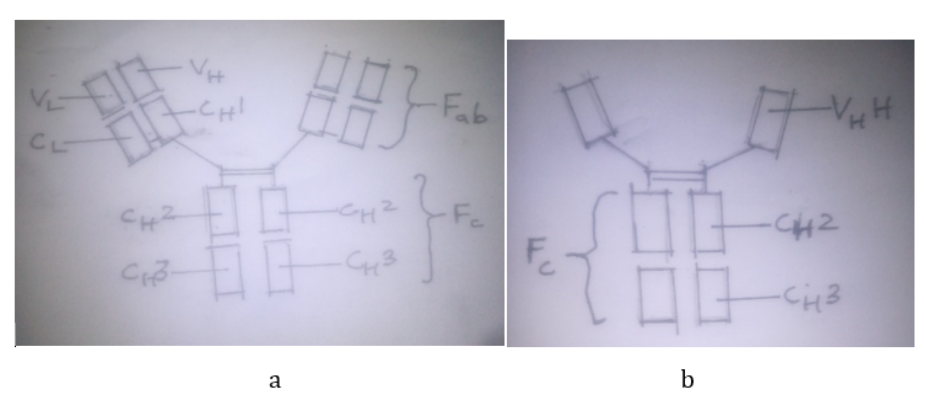
A typically tested method for production of sybodies [14,53,82]:

1. *In vitro* selection of sybodies from phage or ribosome display for generation of conformation-specific binders against labile membrane proteins or protein complexes.
2. Characterization with reference to target antigen and select single clones of sybodies.
3. Sequence the clones of sybodies to help monitor efficiency.
4. Determine the expression patterns from generated sequence data.
5. Purify qualified sybodies.
6. Now ready to play in immunotherapy engagement domain.

Methods for purification of sybodies:

- Chromatography [14] - by Affinity or Size exclusion method.
- Gel filtration [83].
- Ammonium sulfate precipitation [84,83] - which can be used to salt-out protein from solution is a first step in antibody purification.

The unique physical and chemical properties of antibodies such as size, solubility, charge, hydrophobicity and binding affinity determine the method to be used for its purification [83].



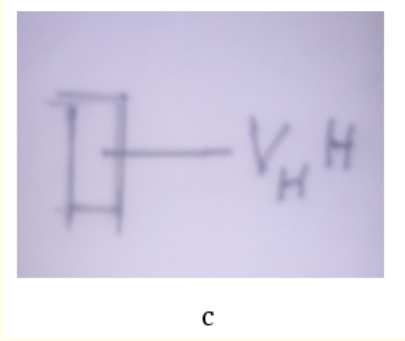


Figure 1: *Two dimensional (2D) structures of conventional antibody compared to 2D structure of Nanobody and Sybody and molecular size details.*

a: Conventional antibody molecule (Approximately 150 kDa).

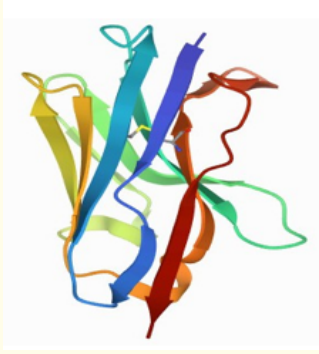
b: Camelid heavy chain antibody (Approximately 95 kDa).

c: sdAb Nanobody molecule (12 - 1 4kDa).

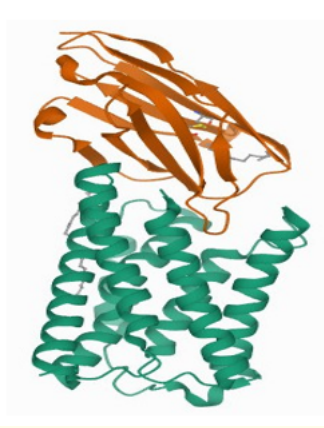
Annotations: *VL, VH: Variable region of light and heavy chains.*

CL, CH: Constant regions of light and heavy chains.

Fab, FC: Sections - Head and tail regions.



Nanobody Wiuf., et al. [58]- Obtained by X-ray diffraction at 1.85A resolution, from a Cameloid antibody raised against KDM5B. Showing 3D crystal structure, retrieved from RCSB Protein Data Bank [85]. Released 2015.



Sybody shown here on top of a target molecule from Braeuer., et al [70]. Showing Nanobody and Sybody folded 3D structure, both capable of penetration to bind on to a difficult to reach target molecule's cleft.

Also released from RCSB Protein Data Bank [86].

Figure 2: *Three dimensional (3D) crustal structures of nanobody (hcabs), perceived "lcabs nanobody" and sybody.*

Applications of nanobodies

This has stemmed from exploitation of its attributes stated earlier for one application of more.

1. Enhancement of capability to recognize and trace target cells in cell microscopy [26,63,74].
2. Enhanced isolation of GFP fusion proteins for biochemical and functional studies [20,22].
3. Enhanced sensing of human prostate specific antigen (hPSA) in diagnostic tests when nanobodies are incorporated into biosensors [20,26,87].
4. It has shown potentials to block interaction of spike protein of SARS-CoV-2 virus with ACE2 receptor on human cells in experimental and non-experimental animal. This is one of the targets for therapy against this virus [6,88].
5. Imaging and diagnostic evaluation of immune cells [27].

Typical nanobdies on clinical trials is Anti-von Willebrand Factor (vWF) nanobody for patients with Acquired Thrombotic Thrombocytopenic Purpura (aTTP) done at over 50 trial health centre locations in United States and aPD1-MSLN-CAR T-cells for treatment of patients with MLSN-positive Advanced Solid Tumours of Lung cancer in China [89], among others.

Applications of sybodies

1. In therapy, they are facilitators for crystallization of target proteins in tough situations like that involving membrane proteins [83]. This is a structure-based drug discovery path and a novel treatment option.
2. Like antibodies, sybodies can be drug molecules in themselves [82].

3. In diagnostics and enhanced structure determination; for instance, sybodies can be used to trap and map intrinsically flexible membrane proteins [57,59].

4. They have been used to effectively neutralize SARS-CoV-2 in various studies [60,90].

5. In the laboratory, they have been used to tag and chaperone target protein function [38,91], stemming from use of their small sizes and offering of more control.

6. They are highly specific binders which have found use for activating or blocking target proteins of interest for therapeutics [38].

7. Sybodies like Affirmers have been employed to track single particles using high resolution microscopy [60].

8. They are used in some diagnostic procedures [14]. Some of these now involve their use to label the most elusive of antigens in tissue samples during immune-diagnostic assays.

9. Typical sybodies on clinical trials is: MP0112 DARPin for Diabetic macula edema (NCT0104267- Clinical trial number) [89], MP0250 Plus Bortezomib+ Dexamethasone in Patients with Multiple Myeloma carried out in over 24 locations mostly in Europe and MP0250 for treatment of Neoplasms being carried out in Spain, UK and Switzerland [92].

Nanobodies and sybodies in cocktail antibody immunotherapy designs and development

Antibody based immunotherapy development to tackle troublesome viral diseases have come on board through engagement of poly-clonal antibodies [141], monoclonal antibodies [40,93], sdAb nanobodies [94,95], sdAb sybodies [97] and now conjugate/cocktail antibody designs [97,98].

Non- natural binding scaffold such as Nbs, DARpin Sybs, affibodies protein molecule constructs that can be as small as 6kDa [99] and immunoglobulin based peptides like neoantigens with small sizes have shown various levels of successes in laboratory *in vivo* and *in vivo* experiments and clinical trials.

A typical instance is that of Abs for treating COVID-19 [100], a good number of which are at various early or advanced stages of clinical trials, such as REGN-CoV2 Ab therapy [101], mAb cocktail Bamlamivimab and Etesevimab which concluded phase clinical trials [102] and received EUA from the US Food and Drug Administration (US-FDA).

The cocktail mAb passed through pre-clinical and clinical tests. In a separate study by Pymm., *et al*. [103], a designed nanobody cocktail named Nanobody-Fc fusion comprising four potent Nbs blocked ACE2 receptor engagement with red blood cell variants present in human populations and neutralized both wild type SARS-CoV-2 and the N501Y D614G SARS-CoV-2 variant at low concentrations of Nb cocktail.

Debie., *et al*. [104] from their study observed that to specifically block easily accessible on-target off-tumour binding sites, a monovalent targeting Nb when co-injected with an excess of its bivalent analogue was found to reduce the high specific extra-tumoural uptake in liver or spleen of a macrophage-mannose receptor-specific Nb, while tumoural uptake remained unaffected [105,104]. This is a promising data towards enhanced utilization of sdAbs as drugs, for drug delivery in form of a nanocarrier (nanomaterial) in therapeutics.

Data from a study by Tijnik., *et al*. [97] showed that fusion of Nb alphaEGFR and albumin alphaAb building blocks results in bi-functional Nb format which is more favourable far therapy as far as pharmacokinetics and tumour deposition are concerned. An insight oriented review by Ozurumba-Dwight., *et al*. [40] in which they authors suggested engagement of antibodies in cocktails with appropriate features that enhance efficacy, good tolerance level and minimal toxicity supports this finding.

Data from a study by Menzel., *et al*. [106] in which they fused Nb to albumin specific Ab resulted in excellent tissue penetration, extended half-life and a positive effect on potency of nanobody therapeutic strength.

The high solubility and modular format of Nbs enables them to be conjugated or linked to other nanobodies [107] in cocktail formats, to generate bi-specific biologics [108] with inclusive improved cellular target specificity.

Biomolecular engineered sdAbs for immunotherapy

The short half-life and occasional low affinity of clones of Nbs [108] for their target antigens can limit their applications [109], including their bioresource capacity. To tackle this challenge, molecular scientists have stepped into bioengineering of sdAbs. For instance:

- From the study of Fan., *et al*. [109], a novel platform named "Fenobody" in which a Nb developed against an influenza virus H5N1 was displayed on the surface of Ferritin in the form of a constructed 24mer. The Fenobody formed clustered bundles which was flexible, thermostable and possessed enhanced affinity for target antigen. The antigen binding apparent affinity of anti-H5N1 Fenobody was increased in folds of some hundreds and the tested half-life was longer than that of anti-H5N1 nanobody. Attributes of solubility in drug action enhances permeability, metabolism and absorption.

The implication on utility of Nbs in Biomedicine is improved attributes for therapeutics, diagnostic imaging and drug delivery, along other biomedical applications.

- Mazzega., *et al*. [110] developed an engineered cross-reacting Nb. Data from this study indicated that this engineered cross-reacting Nb is useful for comparative anatomy.
- Nanobodies are conjugated into branched or linear polyethylene glycol (PEG) [111].
- Nb fusion with Albumin alpha-Ab building block domains [97].
- Nb fusion with Fc fragments of IgG [112].
- Developing multivalent Nb fusion constructs [113] from Nb-species like Mouse/Monkey/Human albumin binding Nb ALB8 in a bispecific or multivalent construction. This can be therapeutically harnessed to deliver drugs. A typical example is the use of this multivalent Nb fusion to deliver a therapeutic protein called "super-oxide dismutase-1" and to deliver therapeutic nanoparticles to healthy and injured tissues [108]. Data from this approach indicated improved avidity for organs tested (spleen and brain), physiologic states and expressed levels of multivalent Nbs.

Metabolism, absorption (uptake) clearance and risk profile of sdAbs in the body (Pharmacokinetics)

In-vivo studies by Debie., *et al*. [104] revealed that monomeric Nbs very quickly accumulate and homogenously distribute through the tumour tissue at a rate that was significantly greater than the dimeric Nb and than mAbs. This implies useful utility for monomeric Nb as tracers in diagnostic imaging procedures and in therapeutics in which they are used to directly target antigens or for delivery of therapeutics. From this study, there was rapid renal clearance of all Nbs used in this study, including fast tumour penetration and absorption at targeted sites. There was rapid renal infiltration of unbound molecules of Nbs during metabolism [106,114].

Studies show that improving affinity of targeting Ab molecule can result in increased affinity and increased retention at target site. In addition, it was discovered that a threshold must be attained to achieve sufficient tumor retention, but enhanced effect from increased affinity is restricted to a certain range, which is also size dependent with smaller sizes in range sizes of around 10 kDa requiring higher affinities of between 0.1 to 10 nM to attain maximum tumor uptake [55]. Debie., *et al*. [104] showed that Nbs conjugated with radio-isotopes

or fluorescent dyes exhibited pharmacological attributes of rapid targeting and fast blood clearance that allows for high specific tumour uptake.

Nbs have been shown to accumulate rapidly into tumors resulting in high tumor -to- background ratio already attained 3 - 5 hours post-injection [115]. In a related study, plasma half-life of non-relevant radio-labelled Nb has been determined to be approximately 2 hours [116], while targeting Nbs reaching tumour sites were observed to be completely eliminated from the blood 24 hours post-administration, with some levels still present in the kidney [117].

Xenaki., *et al*. [115] revealed that parameters such as affinity, valency, antigen density, antibody metabolism and half-life extension are considered when choosing appropriate Ab based targeting agents to be used in immunotherapy. Smaller sized agents can have some advantages over larger ones [55].

The study by Tijinik., *et al*. [97] (2008) on a Nb alphaEGFR fusion conjugated with Nb alphaEGFR to form a dimeric Nb, or this same dimer conjugated with conventional alphaAb, revealed the following:

- Rapid blood clearance of dimeric alphaEGFR-alphaEGFR dimeric Nb radio-labeled with Lu. This caused low tumour uptake.
- Introducing conventional Ab of alphaAb into the conjugate resulted in a significant slower blood clearance and higher tumour uptake that was faster and deeper than when alphaAb was used alone.

As such, based on this observed pharmacokinetic and tumour penetration character, diligently constructed simple fusion alphaEGFR-alphaAb cocktail of Abs produced bi-functional Nb that is more favourable for therapy.

Studies and available data on Pharmacokinetics of Nbs indicate that Nbs and Nb-based biologics are highly specific and have well understood turnable *in-vivo* pharmacodynamics with little, if any toxicity, while its metabolism is non-toxic just and biodegradable with adjustable *in-vivo* half life [81,106]. Also, their predecessor mAbs are biodegradable and found use in immunotherapy [106] but the dimensions attained by sdAbs in this attributes bring in added perspectives for design and development of impacting therapeutics to support our already existing portfolio of immunotherapeutic-techniques and tools for this. The half life has been adjusted by PEGylation, fusion to other conjugates or by monomer Nbs.

It is worth noting that the small sizes of sdAbs enhances rapid clearance from the body, making them rapidly metabolized and absorbed by the tissues of the body, while they show more rapid dissociation kinetics than traditional Abs [106].

Since it has been established in pharmacological principles that when diffusion rate of Ab molecule delivery into target site is faster than clearance (Thiele modulus less than 1), the moving Ab front will successfully reach the core of the tumour [115]. As such, this should be put into consideration when improving pharmacokinetic properties of an Ab molecule. This Thiele modulus describes the ratio between internationalization rate and diffusion/binding rate, which is known in pharmacology to determine whether the administrated Ab targets the whole tumour.

Interaction of sdAbs with cell receptors and cellular components

sdAbs have been shown to be potent reagents to stabilize mechanistic (membrane integrity framework) and structural attributes of membrane proteins [96]. This enabled study of interactions between Syb molecules targeting cell membrane proteins and between Syb molecules-target pathological cell membrane protein moieties.

What then are the applicational benefits of enhanced understanding of these two interactive phenomena? According to Ahuja., *et al*. [118], it will:

1. Facilitate diagnostic target imaging, drug delivery via sdAb Syb or sdAb Nb nanocarrier platforms.

2. Facilitate nano-therapeutic use of such Sybs.

3. Zimmerman., *et al*. [96], Soave., *et al*. [119] and Rossoti., *et al*. [120] in their separate studies showed use of Nbs conjugated to synthetic ligands to improve cell surface receptor selectivity as crystallization chaperons for structural studies of membrane proteins and use in imaging through tracers.

4. Nbs are widely used to target soluble protein antigens or those found on surfaces of cells [121,122]. This will be useful for structural studies and in diagnostic imaging.

Creation of novel nanobodies

In the quest to create more novel Nbs from existing natural forms and basically from Camelid species and already existing formats from sybodies, some featured methods that have proved successful include:

- Mutagenesis to increase diversity of Nbs [123].
- Virus mediated directed evolution [122,124].

Discussion

The unique attributes of sdAbs (Ns abd Sybs) which includes nano-scaled ultra small sizes as severally extracted and presented from various studies in this review, high solubility, high tumour and target pathologic cells uptake absorption, modular nature that enhances conjugation to bio-molecular moieties as presented here, enhanced and adjustable half-life and affinity, all combine to enhance the utility of sdAbs in directed target therapeutic action on target antigens and sites and for drug delivery in form of Nanocarriers. Also, they serve as tissue function modifiers, are engaged as tracers in imaging diagnostic procedures and act as chaperons with high specificity and selectivity for membrane receptors, an attribute that support use in therapeutic designs and applications. Small molecular sizes of biomolecules enhances tissue penetration and distribution of Ab fragments and efficacy of Ab based drugs [125,126]. Data from several sources indicates improved tumour penetration and single cell targeting of Antibody-Drug conjugates increases anti-cancer efficacy and host survival as exposited in studies by Cilliers., *et al*. [127] and D'Huyvetter., *et al*. [128], among others.

When small targeting molecules are to be engaged in therapeutics, modifications in order to improve half-life and binding affinity are required. For instance, longer half-life enhances longer residence time in tumour areas during targeting and more homogenous distribution in the tumour expanse.

Small sizes interplay well with conjugation with enhanced support for half-life extension [115,129]. Longer half-life increases therapeutic index of drug molecules [115].

Immunohistochemical analysis showed increased size and cell binding but decreased tumour penetration for tested antibody from studies by Muchekehu., *et al* [126]. High affinity range required for high tumour penetration by small size Abs can cause less homogenous distribution compared to lower affinity Abs [130].

Small sizes have better diffusion capacities into tumours and rapidly accumulate within its growth and pathologic domain, with added different binding properties in conjugates which helps reduce binding site barrier effect [55,131,132].

Properties of Ab molecules used to target pathogen or expanse of deranged cells of cancer in the body, incorporated attributes of size, shape and charge and the vasculature and its pore sizes through which it diffuses [55,115].

There is an inverse relationship between size of therapeutic molecule used to target the site and the available vasculature permeability [132].

The classical and crystal structures of Nanobody and Sybody bio-molecules with attributes that enhance specific binding to conformational epitopes on antigenic or target tissue sites is a positive attribute that enhances their capacity to elicit appropriate therapeutic or protective functions upon binding, to support drug action. For instance, Ankyrin repeat Sybody modules assemble into elongated and slightly curved structures to present specifically shaped surface that makes precise contacts with its binding partners. This aids their engagement to deliver drugs and bio-molecules in "bind and fit-in conformations" at their desired pharmacological targets in augmenting clinical applications to treat diseases. Coupled to the benefit from the structural features of sdAb Nbs and Sybs, is their small size that is beneficial in reaching difficult to access epitopes on target molecule or antigens.

Monoclonal antibodies which have been predecessor antibody immune-molecules, prior to discovery of sdAbs, have showed immnunogenic and immunotherapeutic attributes that enabled their being engaged to target pathogens and cancer cells [40,101,102,133,134]. In addition, mAbs have been engineered to deliver therapy through conjugational devices [15,44,45] and for diagnosis [46,48]. This provides clues that sdAbs have come in to support mAbs and other forms of conventional Ab therapy that form Ab immunotherapy based pool, before entry of sdAbs like Nbs and Sybs.

For these nanobodies to be effective therapeutically against tumour cells, longer time interval is required between two consecutive administrations of lower doses of nanobodies in order to maintain high loads of nanobodies at target site [5,109,111]. The development of Ab conjugates and cocktails is now tackling this issue with some good level of successes, as presented in this review, from past literature and recorded data.

Studies have revealed the modular nature of sdAbs [107,134] which enables them to be conjugated to other Nbs [98,106,107] or bioengineered with certain biomolecules such as Ferritin while retaining their flexibility [109] that is suitable for spatial structure based antigen or peptide biding. Also, they feature enhanced half-life [97,109,135] small size enhanced solubility, permeability, absorption into tissues such as tumour cells and affinity for binding as earlier presented in this review.

Supporting their therapeutic capacity in conjugated cocktail Ab bioengineered formats is their retaining ultra-small sizes which enables them to be used as nano-carriers (deliverers) of drugs, toxic molecules and other forms of therapeutics to target sites in clinical applications.

The "binding site barrier effect" was attributed to the contributions of Fujimori's group in Japan and states that the higher affinity Abs might show restricted penetration into the tumor mass, as a result of their binding to surface located receptors [120,130]. What is the logic and reality behind this? As thes Abs bind onto cell surface receptors, it enhances further diffusion of unbound Abs, which delays penetration by unbound Ab molecules into target cells and site. Therefore, just like the Thiele modulus based analytic effect, Ab concentration must be enough at saturation concentration levels to maximize and optimize "binding site barrier effect".

The risk profile of sdAbs (Nbs and Sybs) based on available data and literature has shown sdAbs to be biodegradable and metabolizes quickly in the body [136] without conversion to products or by-products at toxic levels that is inimical to human health. Recent advances shows that linkage of specific dyes to sdAbs can be tailored to enhance targeted delivery into tissues for improved imaging in diagnostic applications [136,137].

Enhanced half-life through bioengineering techniques is a supportive attribute in the pharmacokinetic profile of Nbs and Sybs which are exploitable in design of appropriate immune based therapeutics.

Creative ideas in sdAb technology enables creation of new and more Nbs, Sybs, Nb-based biologics and Sb-based biologics with avenues for improved and enhanced biological and pharmacological functions and profile to bring in more tools for clinical therapy. It is worth noting that these products are also subjected to rigorous pre-clinical and clinical evaluations based on air-marked yard-sticks of drug development regulation bodies like the US-FDA [138] and the United Kingdom Medicines and Healthcare Products Regulatory Agency (UK MHRA) [139], to ensure that the sdAb biologics which scale through these tests move from the molecular and pharmacological laboratory bench-sides to the clinical application bedsides, in our health care centres. The clinical tests are rigorous and involves Phases 1 (first time test in small group of people for save dosage range and safety), II (continued test on larger groups for side effects) and III (conducted on larger populations in different countries and regions for efficacy and any other side effects from treatment before EUA approval could be given). Stage IV clinical trials is for further studies conducted in wider populations over a longer timeframe, after country approvals have been given for EUA from phase III clinical trials [140].

Nbs and Sybs in their single unit and bioengineered conjugated formats, have been found to be vulnerable to selective target soluble protein and antigens and cell surface membrane proteins [96,118,120,122], thus providing platforms for interactions that stabilize mechanistic membrane integrity framework [54] and structural depths of membrane proteins to provide non-pharmacological benefits in area of diagnostic imaging [54,104,136]. Engagement of ultra-structural studies is of benefit to the body of knowledge on potentials of sdAbs for present and future utilities of pharmacological benefits to deliver therapeutic biomolecules to target organs and tissues in the body in added clinical applications.

It should be noted that the mechanistic and structural studies of membrane proteins require the stabilization of specific conformation and sdAbs are potent reagents (biomolecules) for this purpose [82,96].

Conformation-specific binders such as Nbs and Sybs raised against membrane proteins have ability to manipulate cells directly at the cell surface and are exquisite tools for basic science and drug delivery [54,118].

In fact, with the technologies developed for design of Sybs, these class of sdAbs can be generated within three weeks or less in standard molecular science based laboratories [96], in support of the new dimensions that sdAbs bring into immunotherapeutic designs and options, in the daunting threats posed by present and emerging diseases.

There is the need for continuous testing of newly created sdAbs and new pool of molecules to be conjugated onto them, aimed at developing new sdAb drugs of optimized potencies in therapy. They will have to be screened for appropriate pharmacokinetic properties to determine and come-up with Ab conjugates having maximized and optimized combinatorial synergistic potentials for therapy against pathogenic and non-pathogenic diseases. These sdAb based drug designs should be at appropriately opitimzed Ab concentrations. Developed sdAb-conjugate drugs has to be in formats that minimize; "binding site barrier effect" which influences extent and depth of penetration of sdAbs and sdAb-conjugate drugs in the expanse of tissue and target sites on one hand and with consideration for effects from the Thiele modules factor which is known to determine whether the administered sdAb conjugate drug does successfully diffuse into the entire target site.

Limitations of nanobodies and sybodies

The small size of nanobodies is beneficial in reaching difficult to access epitopes on target molecule or antigens. However, this same small size has been found to be within the range (molecules of molecular weight below 50,000 kDa) of small molecules that the kidney glomerular filtration process is able to rapidly clear out of the body system. For these nanobodies to be effective therapeutically against

tumour cells, longer time interval is required between two consecutive administrations of lower doses of nanobodies in order to maintain high loads of nanobodies at target site [54]. This challenge has witnessed steps and approaches to tackle it as presented in this review.

Conclusion

In the light of the foregoing, key biophysical and chemical properties of Nanobodies and Sybodies have provided impact on these immunological based molecules in providing roles in drug discovery, drug delivery tools, diagnosis, enhanced cell structure studies and in precision medicine where specificities are watch words. They are adding support to conventional antibodies (like monoclonal antibodies) in bio-molecular tool options for use in molecular techniques based Pharmaceutical drug target discoveries and development. Clinical trials evidence based data, gives clues of the reality of research in these novel immune-molecules for immunotherapy. There are rooms for consolidations, improvements and more robust discoveries in this area of immunotherapy research.

Synergistically acting nanobodies and sybodies will be interesting for studies and trial engagements for their application-based effects, with bio-computational modeling support.

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