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IgY technology: Methods for developing and evaluating avian immunoglobulins for the *in vitro* detection of biomolecules

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Abstract

The term “IgY technology” was introduced in the literature in the mid 1990s to describe a procedure involving immunization of avian species, mainly laying hens and consequent isolation of the polyclonal IgYs from the “immune” egg yolk (thus avoiding bleeding and animal stress). IgYs have been applied to various fields of medicine and biotechnology. The present article will deal with specific aspects of IgY technology, focusing on the currently reported methods for developing, isolating, evaluating and storing polyclonal IgYs. Other topics such as current information on isolation protocols or evaluation of IgYs from different avian species are also discussed. Specific advantages of IgY technology (*e.g.*, novel antibody specificities that may emerge *via* the avian immune system) will also be discussed. Recent *in vitro* applications of polyclonal egg yolk-derived IgYs to the field of disease diagnosis in human and veterinary medicine through *in vitro* immunodetection of target biomolecules will be presented. Moreover, ethical aspects associated with animal well-being as well as new promising approaches that are relevant to the original IgY technology (*e.g.*, development of monoclonal IgYs and IgY-like antibodies through the phage display technique or in transgenic chickens) and future prospects in the area will also be mentioned.

Key Words: Animal welfare; Polyclonal IgYs; Egg yolk; IgY technology; Relevant-to-IgY-technology approaches; *In vitro* immunodetection techniques

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Core Tip: IgY technology has been widely used during the last decades, especially as a means for the efficient *in vitro* immunodetection of biomolecules in various fields of research and disease diagnosis. Despite the very promising relevant new approaches,

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there is still space to further exploit the original IgY technology due to functional, practical, and ethical reasons/advantages associated with the unique features of IgYs, the highly efficient isolation of large amounts of IgYs from the immune egg yolk, and the avoidance of animal bleeding, respectively.

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INTRODUCTION

The term "IgY technology" was introduced in the 1990s to describe a procedure consisting of immunization of birds, especially laying hens, in order to produce polyclonal antibodies of the Y class (IgYs). IgYs can be isolated in large quantities from "immune" egg yolk (thus avoiding the animal bleeding procedure, which is stressful for an animal) and has been applied to various fields of biotechnology and biomedicine[1-3]. To date, IgYs developed in poultry and isolated from the egg yolk as aforementioned have been and are still being used as specific laboratory tools, especially for detecting biomolecules in biological specimens through various *in vitro* techniques (and also as *in vivo* immunotherapeutic agents).

The origins of the IgY technology can be traced back many years, *i.e.* at the end of the 19th century, when Klemperer observed that immunized hens (*Gallus domesticus*) generated antibodies that were present in the egg yolk[2-4]. Subsequently, a new type of immunoglobulin was found in the blood and egg yolk of birds (also in lungfish, amphibians and reptiles), which was called IgY[3,5]. Actually, birds, which do not produce colostrum like mammalian organisms do, use the yolk of their eggs as a very effective source of antibodies through which they can transfer humoral immunity to their offspring, until the latter develops fully mature immune system[6]. Transfer/accumulation of IgY from blood to/in the egg yolk, which is realized by a selective transport mechanism in avian mature oocytes and mediated by specific receptor(s)[7-9], enables the non-invasive isolation of antibodies and eliminates the need to bleed the animal. Isolation and subsequent application of egg yolk-derived antibodies minimize animal suffering and this meets at least one of the three main requirements for animal welfare, *i.e.* "Reduction," "Replacement," "Refinement," as they have been summarized in the "3Rs principle"[10]. As a consequence, in 1996 the European Centre for the Validation of Alternative Methods to animal testing (ECVAM) strongly recommended avian antibodies as alternative to mammalian ones[1]. In parallel, in the mid 1990s the term "IgY technology" was introduced in the literature, as already mentioned; in 1999, the IgY technology was approved as an alternative method for supporting animal welfare by the Veterinary Office of the Swiss Government[3].

Egg yolk is composed mainly of water, which accounts for approximately 50% of its weight, and contains many important nutrients and preservatives, since it serves the role of a protective chamber for the hen embryo. The dry weight of egg yolk is composed mostly by lipids (67%) and also proteins (33%). Egg yolk proteins are distributed between granules and plasma, in which granules are suspended. Granule proteins are divided into α - and β -lipovitellins (70%), phosvitin (16%), and low-density lipoproteins (12%), whereas plasma proteins include α -, β - and γ -livetins and low-density proteins[11]. A precursor of the major egg yolk proteins is vitellogenin, consisting of vitellogenin I (molecular weight [MW]: 260 kDa), vitellogenin II or major vitellogenin (MW: 246 kDa), and vitellogenin III (MW: 210 kDa)[12-14]. IgYs, which are the main constituent of γ -livetins, are among the most important and most abundant egg yolk proteins[11].

IgY is considered to be the functional equivalent and evolutionary precursor of mammalian IgG and probably of mammalian IgE[15]. Due to this functional and evolutionary relationship, some researchers use the term (avian) IgG instead of IgY; however, the first articles in the field have put emphasis on the distinct differences between IgG and IgY and strongly suggested use of the term IgY[5]. In addition to IgYs, there are two more avian immunoglobulin classes, avian IgM and IgA, which are

similar to mammalian IgM and IgA. Mammalian equivalents of IgE and IgD have not been found in hens[16].

Like mammalian IgG, IgY is composed of two heavy (H) and two light (L) polypeptide chains, which are organized in the Y-shaped characteristic “unit,” and contains two identical binding sites for the antigen. However, the structure of IgY is actually different than that of IgG and this results in distinct properties, as well. The nucleotide sequence corresponding to the hen epsilon (“v”) heavy chain has revealed that the molecule contains four constant and one variable Ig heavy chain domains; the additional domain (Cv2) has been conserved in mammalian IgE, but “transformed” into the flexible hinge region in mammalian IgG. As a consequence, the IgY molecule has higher molecular mass (approximately 180 kDa), than mammalian IgG (approximately 160 kDa). Moreover, the Fc part of IgY has a different carbohydrate content compared to the Fc part of IgG. An intact Fc part is necessary for the transfer of IgY from blood serum to egg yolk. In ducks an alternatively spliced form of IgY, the so-called IgY ΔFc, is also present. This variant lacks the Fc region and is mainly found in the blood serum. Hen as well as ostrich and pigeon express only the full-length version of IgY. In some birds, including hen, duck, zebra finch and ostrich, only a single κ light-chain locus has been found. The bursa of fabricius is the site in which immature B-cells are differentiated into mature and competent B-cells, while the spleen is the organ in which plasma cells, *i.e.* the antibody-producing cells, proliferate and memory cells are located. IgY’s heavy and light chain loci consist of single functional V, D, and J genes; in addition to the single functional V genes, there are several pseudo-V genes that lack the usual transcription-regulatory and signal-recognition sequences and are not functional. The antibody diversity in avian organisms is mainly achieved by the so-called gene conversion, through which 10 to more than 120 base pairs from not functional pseudo-genes are transferred to the functional V gene[3,16,17].

The distinct structural features of IgY offer several functional advantages to this unique immunoglobulin type, rendering IgY a versatile and invaluable *in vitro* tool in biotechnology research and in disease diagnostics. Moreover, many reports have suggested *in vivo* application of IgYs in various fields of immunotherapy. The advantages of IgYs include: high potential for developing specific IgYs against conserved mammalian proteins due to the evolutionary distance between mammals and birds, avoidance of activating the mammalian (including human) complement system and reaction with mammalian Fc receptors, ability to isolate substantial amounts of IgYs from immune egg yolks, and avoidance of animal bleeding, which fulfills the “refinement” ethical requirement, as already mentioned[3,18,19].

In the last several decades, more complicated technologies associated with the original IgY technology have emerged, such as the development of avian monoclonal antibodies *via* hybridoma and recombinant techniques, mainly through the phage display technique[20]. Although the above antibodies are IgYs (or IgY-like) immunoglobulins and therefore have all (or part of) the consequent advantages, they are isolated from the supernatant of suitable cell cultures and are not egg yolk-derived. Thus, strictly speaking and at least in our opinion, the techniques leading to the development of monoclonal IgYs cannot be classified as a part of the original IgY technology. On the other hand, transgenic chickens[21] have been used for the production of recombinant proteins, including recombinant antibodies (mostly human/humanized ones), which can be isolated mainly from egg white and are recommended especially for *in vivo* therapeutic applications. Though the aforementioned antibodies have not gained wide application yet and their development and evaluation are considered outside the main scope of the present article, they are considered very promising and will be briefly presented.

The present review article will focus on specific aspects of the original IgY technology, such as immunization of laying hens, isolation of the IgYs developed from the immune egg yolk and consequent immunochemical evaluation. Various recent applications of polyclonal IgYs to the *in vitro* immunodetection of various biomolecules will be also presented and discussed.

DEVELOPMENT AND EVALUATION OF EGG YOLK-DERIVED POLYCLONAL IGYS

General aspects

IgY technology has produced a large number of valuable immunochemical tools for biotechnology and medicine since the 1990s. Various parameters that are associated

with and can affect the results of the IgY technology have been reported in the literature such as housing and breeding conditions, line, age, and stage of development of the immunized birds[2,3,18,22]. Laying hens are the avian organisms of choice (*e.g.*, White Leghorn and Rhode Island Red hens) and are used for immunization to produce polyclonal IgYs throughout their egg-laying period. Other types of poultry such as duck, goose, ostrich, and quail have been referred to in the literature, though to a lesser extent[23-26]. Normal hen lines and conventional housing, *e.g.*, in suitable cages[27], are usually adequate to produce IgYs for research purposes; however, when the IgYs are to be applied as human therapeutics, the use of specific pathogen-free hens is considered necessary[1,3]. Administration of specific food supplements during hens' breeding, *e.g.*, carnitine, has been proposed in the literature as a means to improve overall yield of IgY production, but the results are often contradictory[28].

Immunizing protocols

Parameters that may influence the immune response include antigen nature and dose, use of adjuvants, route of administration, and overall immunization schedule[3].

Both, complex antigens, *e.g.*, whole viruses, bacteria and parasites[29-33] and individual biomolecules, *e.g.*, large proteins[34,35], or small peptides conjugated to a suitable carrier protein, such as keyhole limpet hemocyanin (KLH)[36,37], have been used to stimulate development of specific IgYs in hens. Our team tried to develop IgYs against various antigens, including a recombinant protein of high molecular mass, *i.e.* human kallikrein-related peptidase 6[38] as well as peptides of the alpha- and beta-thymosin families isolated from mammalian tissues or synthetically prepared, either conjugated to KLH or not[39-41]. Moreover, we successfully developed IgYs against the olive fruit fly pheromone by using a KLH-conjugate of the synthetic hapten (\pm)- β -[3-(1,7-dioxaspiro[5.5]undecane)] propionic acid[27].

The antigen dose may be also critical, since too much or too little antigen can lead to an undesirable immune response[2]. Different antigen doses have been reported in the literature. In an early study, a good immune response in hens immunized with bovine serum albumin at doses as low as 0.1-1.0 μ g was reported[3]; however, higher doses ranging from 10 to 1000 μ g (most often 50-100 μ g) have been also used. Information on the doses administered to immunize hens has been presented in a recent review[18].

The outcome of immunization is commonly enhanced by the addition of adjuvants, though successful immunization of hens without any adjuvant has been reported in the literature[3]. Among the adjuvant preparations that have been described till now, Freund's complete adjuvant (FCA) is still considered the gold standard for generating high levels of antibodies in animals, including birds. FCA is a suspension of heat-killed and dried mycobacteria (*Mycobacterium spp.*) in mineral oil, which forms a depot at the injection site and slows down release of the antigen in the host organism, so that long-lasting exposure and a non-specific immune stimulation is achieved. The main problem of FCA is the severe tissue damage it causes at the injection sites, which is usually attributed to the mycobacteria it contains. Although a few studies have reported that hens can better tolerate FCA, in comparison with mammals, other studies have reported contradictory data. For this reason, Freund's incomplete adjuvant, *i.e.* Freund's adjuvant without mycobacteria, is commonly used for booster injections as an alternative to FCA, which is used only in the first immunization[18]. Use of other adjuvants has been also reported in IgY technology, such as the so-called, mineral-oil based Montanide adjuvant, along with oligodeoxynucleotides containing C-phosphate guanosine motifs, which are promising immunoenhancing agents[28]. Research in the area of developing new adjuvants, both highly efficient and animal welfare-friendly, is being continued.

Regarding the route of administration, several approaches have been tested. The most recommended one is the intramuscular injection (i.m.) into the breast tissues[3, 29,34,42] in multiple sites; i.m. administration in the thigh muscle has been also used but according to some reports it may cause lameness and has to be avoided[18]. Subcutaneous (s.c.) immunization in the neck has also been used by several research teams including our team[27,38,39]. As reported, i.m. immunization in breast muscle is most suited especially for young hens[18]. The intravenous (i.v.) route has been very rarely used, without adjuvants and at a very slow injection rate. The intraperitoneal (i.p.) route, which Klempner has followed in his pioneer work, is hardly used these days. Efforts to immunize hens orally have been also reported[3,30,43].

The interval between the first and second (*i.e.* first booster) immunization is considered a critical parameter in hen immunization protocols. Age of hens when first immunized might also be an issue. However, literature information on these specific parameters substantially varies. A general recommendation is to administer a booster immunization when the IgY titer reaches a plateau or begins to decrease[44]. If a

substantial decrease in the antibody titer has been observed, further immunizations can be performed during the entire laying period, which lasts about 72 wk[22], to keep the antibody titer adequately high for as long as possible, in many cases for more than 150 d[18]. As presented in a previous review[3], some immunization protocols have recommended antigen administration at days 0, 14 and 28, or once a week for 7 consecutive weeks, or at day 0, week 10, and week 15. Other protocols propose hen immunization at 10-d intervals, but in most cases, the interval between the first and second immunization is at least 4 wk, while another protocol has reported achievement of a high antibody titer by prolonging the boost interval from 14 to 42 d. Intervals among booster injections also vary, averaging 2 wk[3]. Our team has mainly used 3-mo-old hens for immunization; the first booster was administered 2 wk after first immunization, while several further injections were given, mostly at 4 wk-intervals[27].

In general, eggs are collected weekly, starting 1 wk prior to the first immunization (pre-immune eggs), eggshells are washed or sanitized with 70% ethanol, and stored at 4°C until further processed for IgY isolation. Lyophilization of egg yolk has also been reported, resulting in an easy-to-mix egg yolk powder with an extended shelf-life[45].

Immunization with plasmids: “DNA-designed” IgYs

Apart from the conventional administration of antigen along with adjuvant, the so-called genetic immunization has also been applied to the production of polyclonal IgYs in avian species[46]. In this context, avian organisms have been immunized with plasmid vectors encoding target eukaryotic antigens, *e.g.*, bovine interferon gamma protein[47], prokaryotic antigens, *e.g.*, *Botulinum* toxin A1[48], as well as viral ones, *e.g.*, antigens from Andes virus[23]; in almost all cases, antibodies Y of desired immunochemical characteristics have been developed. A great deal of effort has been put forth to improve DNA-vaccine delivery, and consequently, immunogenicity. The “gene gun” method has garnered much attention, since low doses of DNA applied *via* a gene gun can efficiently induce high antibody titers against the antigen encoded[49]. Although DNA immunization is a promising approach, which prevents costly and tedious preparation of purified antigens or presence of adjuvants in the immunization mixture, it has not yet gained wide application.

Isolation of IgYs from the egg yolk

Hen eggs are an excellent source of high amounts of antibodies[19]. An average hen can lay roughly 325 eggs a year. Given that according to the literature an egg can produce 60-150 mg[50], or 40-80 mg total IgY per egg yolk depending on the hen’s age [22], one hen can roughly produce 20-40 g of antibodies a year, with 1%-2% up to 10% of the antibodies being antigen-specific[18,51], which is much higher than that obtained from mammalian sources[11].

Isolation of IgYs from the “immune” egg yolk in pure form is a challenging task. Several protocols have been described, with different characteristics in terms of total yield, purity, duration, convenience, and cost[42]. IgYs account for about 3%-5% of the egg yolk proteins, which are dispersed in a lipid emulsion combined with lipoproteins and glycoproteins. Consequently, in most cases, IgY isolation involves, first, removal of lipids to form a water-soluble fraction (“de-lipidation” step), and then precipitation of the antibodies that are present in the water-soluble fraction with various approaches[3,18].

The most commonly used de-lipidation technique is the “acidified water dilution method” [52], using 6- to 10-fold dilution of egg yolk in water at pH ~5, incubation for several hours at 4°C and then centrifugation, at the end of which the lipid portion is precipitated and the water-soluble portion is collected in the supernatant. Alternatively, lipid removal has been successfully performed by means of organic solvents (chloroform, acetone, isopropanol)[53,54], acids (caprylic acid, trichloroacetic acid)[55] or natural gums (polyanionic polysaccharides, *e.g.*, xanthans)[56]. A de-lipidation solution containing polysaccharides (such as pectin, λ -carrageenan, carboxymethylcellulose, methylcellulose, and dextran sulfate) has been also reported[57].

After de-lipidation, various IgY extraction methods that can be applied either to laboratory- or to large-scale production have appeared in the literature; these methods can be divided into three main groups, *i.e.* precipitation, chromatographic and filtration methods.

Precipitation methods, involving precipitation of IgYs with saturated salt solutions, such as ammonium sulfate, sodium sulfate or sodium chloride[58,59], polyethylene glycol (PEG)[60], caprylic acid[61,62] and carrageenan[63]. PEG precipitation usually involves, first, dilution of egg yolk in phosphate-buffered saline (PBS) containing PEG 6000 at low concentration (3.5%), to facilitate de-lipidation. After centrifugation, the

supernatant is treated with 8.5% and then with 12% PEG 6000 to precipitate IgYs[30]. Among the above methods, ammonium sulfate precipitation is considered one of the best choices for the scale-up purification of IgY[11], with most suitable concentration of ammonium sulfate being 20%[55]. Extracted IgY samples usually undergo a final dialysis step, usually against PBS, to eliminate residual salts from the extraction procedure.

Chromatographic methods include low-pressure chromatography[30], ion exchange chromatography[52,59], high-resolution chromatography through multicolumn systems[64] and affinity chromatography[65]. Conventional affinity chromatographic methods using protein A or protein G columns cannot be performed for IgY purification, since IgYs, contrary to IgGs, do not bind to protein A or G[66]. Other types of ligands are therefore required, such as the elastin-like polypeptide-tagged immunoglobulin-binding domain of streptococcal protein G[67]. Still other ligands, such as IgY-binding peptides screened from a random peptide library, have been also proposed as a means of IgY purification[68]. IgY can also be purified with thiophilic adsorption chromatography, usually through commercially available IgY-extraction columns[18,69]. However, chromatographic techniques are generally expensive and impractical for the large-scale production of antibodies, while they have not proven to substantially increase purity of the final product when compared with simple precipitation methods, such as ammonium sulfate precipitation.

Filtration methods, such as ultrafiltration[52,70], have also been used as IgY extraction methods.

As reported, a combination of the aforementioned methods, *e.g.*, a combination of PEG precipitation with affinity chromatography[22] or ammonium sulfate precipitation with ion exchange chromatography[59], can further increase the purity of the IgY preparation. Moreover, sequential precipitation with 31% ammonium sulfate and 12% PEG resulted in IgY antibodies of more than 95% purity without any loss in immunoreactivity[64].

Despite the numerous protocols described in the literature, the most popular isolation strategy of IgYs from immune eggs involves a de-lipidation step, in which IgY is extracted in the supernatant after treating the egg yolk with 10 volumes of acidic water and a subsequent precipitation step, in which IgY precipitates with ammonium sulfate or PEG, at suitable concentrations[30].

Storage

According to the literature, after their isolation, IgYs can be stored for long periods (from a few months to a few years), preferably at -20 °C[22,71], since they are considered reasonably stable biomolecules, like mammalian IgGs[72]. IgY is stable at pH 4-9 and up to 65 °C in aqueous solutions. The addition of stabilizing reagents or high concentrations of salts can further increase resistance of the IgY molecule; *e.g.*, heat stability could be increased up to 70 °C by the addition of sugars, such as 30% sucrose, trehalose or lactose[3]. Useful information concerning earlier findings on the stability and storage conditions of IgYs has appeared in recent review articles[73]. Freeze-drying has been used to facilitate long storage of IgYs[74], though some researchers have reported that freeze-drying may lead to some loss of antigen-binding activity of IgY[45]. Lyophilization of proteins, including IgYs, induces freezing and dehydration stresses, which may result in protein structural changes or even unfolding [75]. Therefore, the addition of cryoprotectants and lyoprotectants has been recommended to protect IgYs during lyophilization[45]. Our team has recently evaluated IgYs that were developed against a KLH-conjugate of the polypeptide prothymosin alpha many years ago and kept as lyophilized powder at -30 °C. As revealed, the IgYs have kept immunoreactivity and were successfully applied to a specific enzyme-linked immunosorbent assay (ELISA) for prothymosin alpha[76].

Evaluation of egg yolk IgYs

Protein concentration: Determination of protein concentration in IgY extracts is usually performed before proceeding to further IgY evaluation. Total protein concentration in IgY extracts has been determined mainly with the Bradford method (indicative references[30,34,35,38,42]), the Lowry method[58] and the bicinchoninic acid protein assay[77]. In addition, protein concentration was assessed with ultraviolet absorption at 280 nm, according to the Lambert-Beer law (indicative references[29,32,33,57,76]).

Purity: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is considered the gold standard technique and has been widely used to assess the purity of the egg yolk-isolated IgYs (indicative references[30,33,34,62,78]). SDS-PAGE

separation under non-reducing or reducing conditions would reveal one or two protein bands, the latter corresponding to heavy and light IgY chains.

Western blotting has been used complementarily with SDS-PAGE to confirm the presence and assess purity of IgYs isolated from immune egg yolks (indicative references[31,33,62,78]). Visualization of the specific protein bands is performed mainly through a color or chemiluminescence development.

In a few cases, additional analytical methods such as high-performance liquid chromatography[57] have also been used to evaluate the purity of IgYs.

Immunoreactivity: The immunoreactivity of egg yolk-derived IgYs is evaluated with well-established immunochemical methods such as dot-blot and ELISA. Dot-blot can be actually considered as a simplified form of ELISA offering mostly qualitative results. Nevertheless, it is a fast, easy, and low-cost technique that may provide useful information and has, therefore, been used by several researchers to evaluate immunoreactivity of IgYs[32,34,36,39,76]. In most cases, however, evaluation of IgY immunoreactivity involves determination of titer against the target antigen through non-competitive ELISAs (indicative references[32,35,45,76,78]). Moreover, other immunochemical characteristics of the isolated IgYs are assessed, such as putative cross-reactivity with various substances through competitive ELISAs (indicative references[31,36,39]). It should be noted that till now and despite the numerous new technologies introduced in the field, ELISA remains the gold standard method for evaluating the basic immunological characteristics of any antibody developed, independently of the antibody class or the production method.

IN VITRO APPLICATION OF EGG YOLK-DERIVED POLYCLONAL IGYS TO THE DETECTION OF BIOMOLECULES

IgY is considered an excellent tool especially for developing *in vitro* methods to detect biomolecules of interest in biological specimens for a series of reasons. First, the evolutionary distance between mammals and birds may facilitate generation of specific IgYs against conserved mammalian proteins, since avian organisms possess a different antibody repertoire than that of mammals and the epitope spectrum of avian antibodies is potentially larger/different than that of mammalian IgGs including novel specificities[19,64]. Second, IgY does not activate the mammalian (including human) complement system and does not react with mammalian Fc receptors; this feature has rendered IgYs an ideal *in vitro* reagent, especially for immunoassays designed to detect biomolecules in human blood serum[64]. Third, substantial amounts of IgY can be isolated from egg yolks; as already mentioned (isolation of IgYs from the egg yolks), one hen can produce 20-40 g of IgY in 1 year, 1%-10% of which is antigen-specific. This advantage of egg yolk IgY is accompanied by other practical superiorities, such as low animal care cost, ease of isolation of antibodies from the egg yolk with simple biochemical methods and overall low production cost[73]. These advantages along with the large-scale facilities currently available render production of egg yolk-derived IgYs, a technically feasible and efficient procedure at industrial level. Some other positive characteristics of IgYs have been reported in the literature, *e.g.*, they can be developed even when hens are immunized with very small amounts of the corresponding antigens[64,71] or that they show higher specificity, binding affinity, and avidity for their targets in comparison with mammalian IgGs[38,73], although other reports have shown controversial data[3]. Last but not least, in the list of IgY advantages is that use of egg yolk IgYs is especially desirable from an ethical aspect of view, concerning refinement of animal experimentation, as already mentioned. Some recent indicative applications of IgYs to the *in vitro* detection of biomolecules (as well as whole viruses/microorganisms) have been summarized and presented in Table 1. Lately, specific IgYs have been developed and used for the immunodiagnosis of pandemic coronavirus disease-2019 (COVID-19)[79], while non-specific IgY has been used to form/visualize the “control line” in point-of-care *in vitro* tests that detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigens[80].

Table 1 *In vitro* applications of polyclonal IgYs

Target biomolecule(s)	<i>In vitro</i> immunochemical technique	Proposed field of application	Ref.
Major surface antigen of <i>Toxoplasma gondii</i> (SAG1)	Latex agglutination assay	Diagnosis of Toxoplasmosis	Cakir-Koc <i>et al</i> [132], 2020
Protein A of <i>Staphylococcus aureus</i>	Immunocapture PCR assay	Detection of <i>Staphylococcus aureus</i> in food samples, skin and nasal swabs	Kota <i>et al</i> [133], 2020
Peptides/proteins present in detoxified western Russell's viper venom	Paper-based microfluidic immunochromatographic test	Differential diagnosis of Russell's viper envenomation	Lin <i>et al</i> [134], 2020
SARS-CoV-2 antigen	Fluorescence immunochromatographic rapid-antigen test	Diagnosis of COVID-19	Porte <i>et al</i> [79], 2020
Antigens present in total saline extract of <i>Taenia crassiceps</i> metacestodes	ELISA	Detection of neurocysticercosis	daSilva <i>et al</i> [32], 2020
Antigens present in total saline extract of <i>Ancylostoma ceylanicum</i>	ELISA	Diagnosis of Hookworm infection	Souza <i>et al</i> [135], 2020
Non-glycosylated synthetic oligopeptides of <i>Dermatophagoides</i> group I allergens	Immuno-dot blot assay (with the use of IgY-colloidal gold nanoparticles conjugates)	Detection of indoor dust mite allergens	Egea <i>et al</i> [136], 2019
Antigens present in whole bacterial suspension of formalin- and heat-inactivated <i>Salmonella typhimurium</i> and <i>Salmonella enteritidis</i>	<i>In vitro</i> immunochemical techniques	Diagnosis of infection with <i>Salmonella typhimurium</i> and <i>Salmonella enteritidis</i>	Esmailnejad <i>et al</i> [26], 2019
Antigenic extracts of <i>Strongyloides venezuelensis</i> infectious filariform larvae and parthenogenetic females	ELISA	Diagnosis of human strongyloidiasis	deFaria <i>et al</i> [33], 2019
Antigens present in total saline extract of <i>Ascaris suum</i> adult life forms	Tissue indirect immunofluorescence assay & ELISA	Diagnosis of human ascariasis	Lopes <i>et al</i> [31], 2019
Free prostate specific antigen	ELISA	Diagnosis of human prostate cancer	Lupicka-Słowic <i>et al</i> [137], 2019
Antigens (capsid proteins VP2 & VP3) present in beta-propiolactone-inactivated enterovirus 71	Fluorescence sensor assay	Diagnosis of hand-foot-and-mouth disease caused by enterovirus 71 infection	Nie <i>et al</i> [138], 2019
<i>Fusarium verticillioides</i> 97K exoantigen	ELISA	Detection of <i>Fusarium verticillioides</i> (and prediction of fumonisin contamination) in poultry feed	Omori <i>et al</i> [139], 2019
Recombinant purified catalytic domain of Karilysin	ELISA	Evaluation of karilysin (<i>i.e.</i> an enzyme secreted by the periodontopathogen <i>Tannerella forsythia</i>) as a biomarker for the diagnosis of periodontitis	Skottrup <i>et al</i> [34], 2019
Fumonisin B1	Lateral flow immunoassay	Detection of fumonisin B1 and fumonisin B2 in maize	Tran <i>et al</i> [140], 2019
Synthetic extracellular peptide of matrix-2 protein of influenza A virus, conserved in all strains	Latex agglutination assay	Diagnosis of infection with Influenza A virus	Budama-Kilinc <i>et al</i> [141], 2018
Sulfamethazine (SMZ)	ELISA, FPIA	Detection of veterinary drug residues (SMZ) in milk	Liang <i>et al</i> [142], 2018
Native calf adenosine deaminase (ADA)	ELISA	Evaluation of ADA as a cancer biomarker	Lupicka-Słowic <i>et al</i> [143], 2018
Nucleoprotein of influenza A virus	Immunocytochemistry, Immunohistochemistry	Diagnosis of infection with influenza A virus	da Silva <i>et al</i> [144], 2018

ADA: Adenosine deaminase; COVID-19: Coronavirus disease-2019; ELISA: Enzyme-linked immunosorbent assay; FPIA: Fluorescence polarization immunoassay; PCR: Polymerase chain reaction; SAG1: Surface antigen 1 of *Toxoplasma gondii*; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SMZ: Sulfamethazine.

RELEVANT APPROACHES AND FUTURE PROSPECTS

Monoclonal IgYs

Since the late 1980s many efforts have been directed toward development and use of avian monoclonal antibodies (mAbs) for research, diagnostic, and therapeutic purposes, because avian mAbs may combine the advantages of avian immuno-

globulins with those of monoclonality, *i.e.* precise characterization and continuous production. Initially, several technical difficulties have emerged; even after technical problems have been addressed and avian mAbs have been produced by hybridomas [81,82], the hybridoma technology has not gained wide application, because it is considered a complex, time-consuming and low-yield process by many researchers. By contrast, antibody-engineering methods proved to be the most frequently techniques used for the production of chicken mAbs. Actually, chicken provides an ideal basis for generating large immune antibody fragment libraries as compared to most mammalian species. In chickens, the large and diverse antibody repertoire is generated by gene conversion, in which segments from non-functional V pseudogenes located upstream are inserted into the rearranged gene, and somatic hypermutation. Since gene conversion has not been observed at the 5'- and 3'-ends of the rearranged gene, it is possible to perform real-time reverse transcription polymerase chain reaction (PCR) of the V-region repertoire with a single pair of primers[20,72]. Of the various recombinant antibody fragments, the full-length single-chain variable fragment (scFv) is the most commonly used. For construction of the scFv antibody library, total RNA is isolated from the spleen cells of immunized or non-immunized chicken and reverse-transcribed into cDNA. Then the variable heavy and light chain domain genes of immunoglobulin antibody cDNA are amplified by PCR and properly assembled to form the full-length scFv fragments, which resemble a functional Fv region. Then the scFv genes are cloned into suitable vectors to construct an antibody-expressing library [83]. Currently, phage display systems are the most often applied recombinant methods for generation and isolation of chicken mAbs[83,84]. In phage display methods, genetically-engineered phages that are capable of displaying recombinant fragments of antibodies on their coat surface can undergo several rounds of biopanning and re-propagation in *Escherichia coli* to enrich for clones exhibiting specific binding. Many IgY-scFv were produced with the phage display method combined with *in vitro* selection technologies, either by research groups[84-87] or companies that provide custom services for the development of monoclonal antibodies Y[88,89]. Among recent technologies reported for producing and isolating monoclonal IgYs is the gel encapsulated microenvironment assay, which is capable of "cross-examining" the entire population of splenic B cells from immunized chickens[90]. In an effort to produce mAbs suitable for *in vivo* administration in immunotherapy, the highly immunogenic constant region of chicken IgYs has been replaced with that of human to generate chicken-human chimeric antibodies[91]. Moreover, humanization of chicken scFvs has been successfully performed using the complementarity-determining region (CDR)-grafting strategy, which replaces human CDRs with chicken CDRs while retaining the human framework region residues, and followed by further optimization when necessary[92,93]. On the other hand, chimeric chicken-mouse or mouse-chicken recombinant mAbs have been produced and their characteristics have been studied[94,95].

Antibodies produced by genetically modified chickens (transgenic chickens)

Over the last decades, significant progress was made in generating recombinant proteins, including mAbs for therapeutic applications, in genetically modified chickens[21,96]. Difficulties in generating modified chickens are mainly attributed to the complex structure of the chicken zygote and the different organization of the chick embryo in comparison to mammals. To successfully generate genetically modified chickens, different methods have been used to achieve stable genomic integration of transgenes and the highest efficiency of germline transmission[97], including direct DNA microinjection into the chicken zygote[98] and use of viral vectors for gene transfer, which is the first applied and considered one of the most successful methods. Thus, the first genetically modified chicken was generated by the insertion of retroviral foreign DNA delivered by avian leukosis virus successfully integrated to the germline[99]. Since then, various viral vectors have been used to generate transgenic chickens for the production of recombinant proteins[100-102] including mAbs[103]. Among these, lentiviral vectors have been reported to offer specific advantages, including ability to transduce dividing and non-dividing cells, a relatively large transgene capacity and the apparent resistance of transduced cells to gene silencing [104]. Lentiviral vectors have been used to introduce transgene constructs comprising suitable sequences from the ovalbumin gene to direct synthesis of associated proteins to oviduct[105]. Despite the fact that the use of viral vectors improves germline transmission, the size limitation of the transgene and the lack of possibility of precise edits remain as drawbacks. One of the most effective approaches to produce transgenic chickens is the *in vitro* transfection of avian cell lines, such as primordial germ cells (PGCs) and embryonic stem cells (ES), the clonal selection and reinsertion

into the embryo leading to fully transgenic progeny in the next generation[106-108]. Following this approach, production of human mAbs in the egg white of chimeric transgenic chickens with the use of genetically modified ES cells carrying ovalbumin expression vectors was successfully performed for the first time; however, although a high amount of functional mAb was produced in the egg white, no transgenic offspring were initially obtained[107]. Heritable transgenic chickens capable of producing mAbs in their egg whites were generated using transfected PGCs with a gene construct designed to express the mAb in chicken oviduct magnum[108]. Specific gene editing of PGCs could be improved using genome-editing tools, such as transcriptional activator-like effector nucleases[109] and the clustered regularly interspaced short palindromic repeats-associated protein 9 system (CRISPR/Cas9 system)[110,111]. CRISPR/Cas9 has been used to generate transgenic chickens for the production of recombinant proteins in the white egg[111], including mAbs[112], or exhibiting resistance to pathogens[113]. Another recent promising approach is the replacement of the chicken immunoglobulin variable regions by human V regions and use of synthetic pseudogene arrays in order to produce affinity matured antibodies in transgenic chickens, called OmniChickens; OmniChicken can thus generate antibodies of basically human sequence, which retain the epitope repertoire of chicken immunoglobulins[114].

DISCUSSION

IgY technology has produced a great number of valuable immunochemical tools for biotechnology and medicine since 1990's. Various parameters that are associated with and can affect the results of the IgY technology have been reported in the literature, such as the immunization procedure. One of the most important parameters is the extraction/purification protocol used for isolating the IgYs from the egg yolk. Several methods of isolation and purification of IgYs from "immune" egg yolks have been reported, as already mentioned; the choice of a specific method depends on several criteria, such as desired yield, purity and final application of the IgYs along with cost and scale of extraction. The most popular isolation strategy consists in a de-lipidation step, in which IgY is extracted in the supernatant after treating the egg yolk with 10 volumes of acidic water, and a subsequent precipitation step, in which IgY precipitates with ammonium sulfate or with PEG, at suitable concentrations[30].

Our team have used the acidified water dilution method followed by precipitation with 19% sodium sulfate[39] or with 8.5% and 12% PEG 6000[27] for the isolation of IgYs from immune egg yolks. SDS-PAGE and western blot analysis of IgYs isolated with sodium sulfate precipitation has revealed a protein impurity with MW of ~35 kDa, which underwent liquid chromatography tandem mass spectrometry analysis and was proposed to be identical with the C-terminal fragment of vitellogenin II precursor protein[39]. The same impurity was also observed by other researchers, who had followed a different isolation protocol involving precipitation with PEG 6000[22]. As later shown[115,116], IgY from hen egg yolk occurs as a complex with peptides, named yolkin, which exhibit immunoregulatory and other biological activity. Yolkin contains several peptides with an apparent molecular weight ranging between 1 and 35 kDa. As reported, purified yolkin constituents are homologous with some fragments of the C-terminal region of vitellogenin II; more specifically, yolkin fractions of MW > 16 kDa are glycoproteins corresponding to the amino acid sequence of vitellogenin II starting at position 1572 aa[12,117]. In our hands, presence of the above impurity did not seem to interfere with the efficiency of IgYs as specific *in vitro* immune reagents.

As already mentioned, egg yolk IgYs have been thought to be superior to mammalian IgGs for *in vitro* applications. The *in vitro* efficiency of IgYs may be questioned only under rare conditions, *e.g.*, due to the putative presence of anti-hen antibodies in biological samples of specific individuals who have been sensitized to hen egg yolk[72]; however, to what extent IgY-specific antibodies may occur in human individuals remains to be clarified. Exempt from the aforementioned few concerns, IgYs are considered ideal and are being continuously developed and used as invaluable *in vitro* laboratory tools up to now (Table 1).

One of the great advantages of the IgY technology is the enhanced probability of generating specific IgYs against conserved mammalian proteins, since hens may exhibit a different antibody repertoire than that of mammalian organisms. With this in mind, our team has immunized hens against the poorly immunogenic, highly conserved polypeptide prothymosin alpha (ProT α , MW: approximately 12 kDa,

isolated from bovine thymus). The anti-ProT α antibodies Y were isolated from the egg yolk and evaluated through dot-blot and ELISA experiments in parallel with antibodies G isolated from the antiserum of rabbits immunized against the same immunogen. As revealed, not only antibodies G, but also antibodies Y showed hardly detectable titer/affinity for ProT α [39]. The above negative result may be attributed to the fact that ProT α is thought to be highly conserved during evolution and ProT α -homologues have been reported in non-mammalian organisms as well[76,118]. Similarly, hens were immunized against the highly conserved polypeptide thymosin beta4 (T β 4, MW: ~5 kDa, synthetic), either conjugated to KLH (T β 4/KLH) or non-conjugated, leading to IgYs of either relatively high titer or, on the contrary, not-detectable titer, respectively[41]. Interestingly, antibodies Y that we developed against a KLH-conjugate of ProT α (anti-ProT α /KLH IgYs) showed high titer and practically no cross-reactivity with a series of ProT α -fragments, including the N-terminal fragment ProT α [1-28] (also known as T α 1), being therefore highly specific for whole-length ProT α , while the corresponding anti-ProT α /KLH rabbit IgGs did cross-react with T α 1[76]. Moreover, when various synthetic fragments of ProT α or T β 4 were conjugated to KLH and used for immunizing hens and rabbits, the results revealed that specific antibodies Y of hardly detectable titer were obtained; on the contrary, rabbit immunization with the same immunogens led to high-titer antibodies G, specific for ProT α or T β 4, respectively[39,41]. The above results support the assumption that novel antibody specificities may emerge *via* the avian immune system and can be obtained through the IgY technology.

Although IgYs for research applications are mainly produced in hens, other birds have also served this purpose, as already mentioned, including duck[23,119], goose [24], quail[26] and ostrich[25], following immunization and isolation protocols similar to those used for hens[18]. Quail, ostrich and other avian species may provide further advantages in the field of IgY technology, such as convenient housing and breeding conditions (quail[26]) or exceptionally high amounts of IgYs obtained (ostrich[25]). Previously, our team has isolated immunoglobulins Y from the egg yolk of several avian species, including ostrich (*Struthio camelus*) and quail (*Coturnix japonica*); the isolation protocol has been developed in-house and based on the acidified water dilution and the PEG precipitation method. Ostrich and quail immunoglobulins Y were characterized in terms of their molecular weight (SDS-PAGE and western-blotting) and their ability to recognize and bind to a commercially available horseradish peroxidase (HRP)-labeled rabbit anti-hen IgY antibody in an ELISA system[120]. As revealed, the ostrich IgYs could be hardly recognized by the HRP-labeled anti-hen antibody we used, though other researchers reported successful use of commercially available secondary anti-hen antibodies to assess the immunochemical efficiency of specific ostrich IgYs[121]. On the other hand, HRP-labeled secondary anti-ostrich-IgY antibodies have been specially developed and used to evaluate ostrich IgYs with ELISA[25]. According to experimental results of ours[120] and others'[26,122], the quail IgYs could be recognized by the HRP-labeled secondary anti-hen antibody, which indicates that quail and hen IgYs may share at least some homology in immunochemically important structural features[123,124]. Wide availability of secondary antibodies for IgYs originated from avian species other than hens will support further expansion of the IgY technology.

In addition to their unequivocal usefulness as *in vitro* immunodetection reagents, IgYs have been proposed as promising *in vivo* therapeutics, *e.g.*, as an alternative to antibiotics treatment against multi-drug resistant or difficult-to-treat pathogens, since they exhibit *in vivo* pathogen-neutralizing activity, especially in mouth, throat, the respiratory tract and lungs[73]. Moreover, since IgYs are not absorbed by the gastrointestinal tube, they have been proposed as perorally administered immunotherapeutics against various viral, bacterial, and fungal infections of the gastro-intestinal tract, especially in veterinary medicine and fish-cultivation[3]; a limitation in wide therapeutic application of perorally administered IgYs is their reduced stability at low pH[72] and several efforts have been made to address this shortcoming. IgYs have been also proposed as locally administered immunotherapeutics for treating skin and other local infections[3]. Lately, specific IgYs have been developed and used for treatment of the pandemic COVID-19[35,64,66,125]. Overall, despite the new promising technologies emerged, literature on the IgY technology continues to expand, encompassing various applications ranging from *in vitro* immunodetection of biomolecules and *in vitro* immunodiagnostics to *in vivo* immunotherapeutics[18,126].

Though development of monoclonal IgYs cannot be considered as a part of the original "IgY technology", it seems very attractive and will probably be the next big step in the area, since it combines the advantages of mAbs with those of avian IgYs. At the initial phase, production of chicken mAbs had to overcome several technical

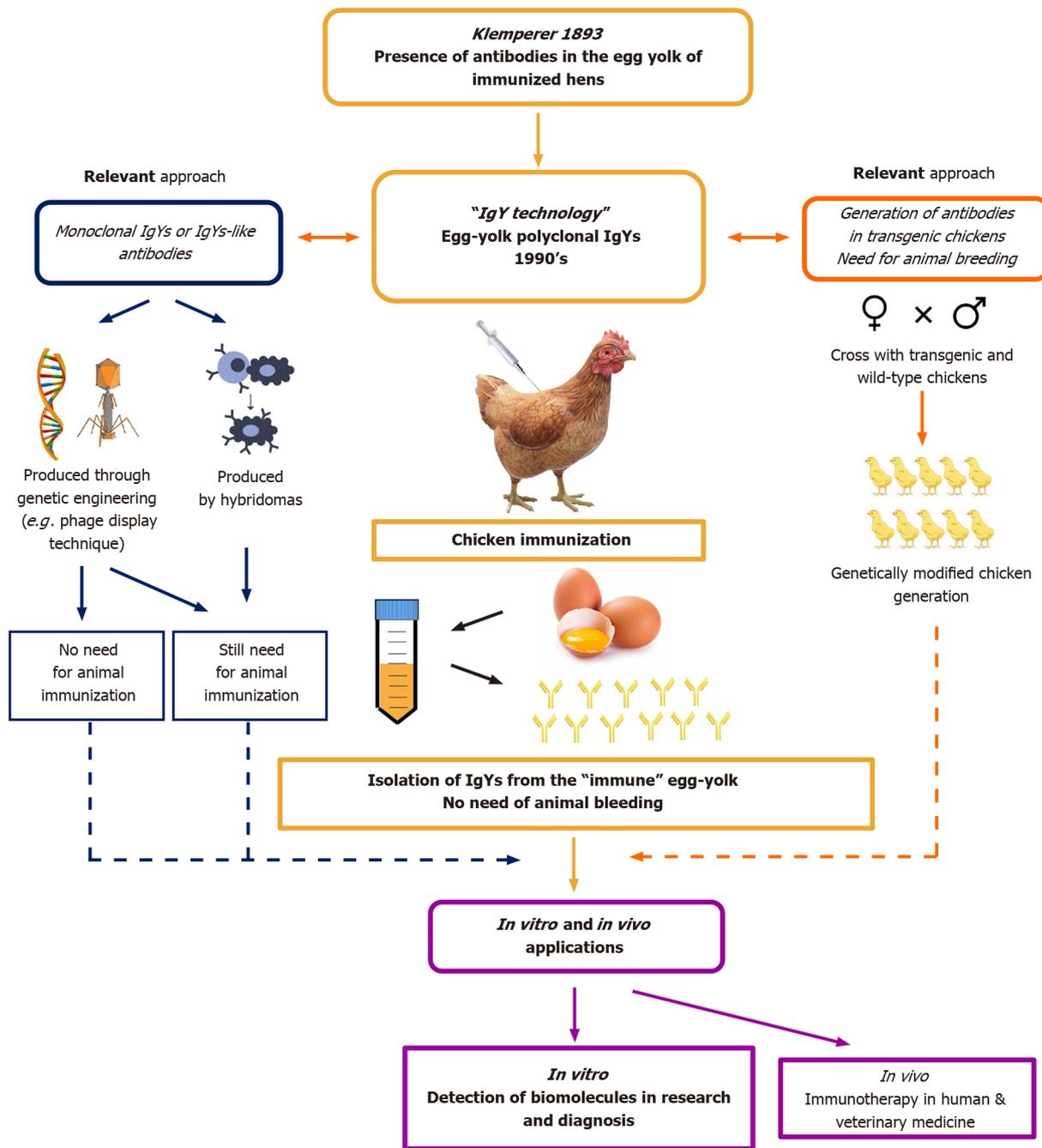


Figure 1 Schematic representation of the main parts comprising the original immunoglobulin Y technology (central axis); promising relevant approaches are also shown (periphery, left and right).

difficulties, including lack of appropriate fusion partners and loss of antibody secreting ability by the hybridoma cells over time[81]; this has been successfully addressed when monoclonal IgYs were generated through combinatorial antibody libraries *via* the phage display methodology[127]. Thus, over the past years, avian libraries have been constructed and several reports on the isolation of avian-derived antibody fragments have been published[20]. The different spectrum of epitopes recognized by the avian immune system may facilitate the development of novel diagnostics, *e.g.*, through targeting highly conserved mammalian proteins, while monoclonality may especially facilitate the development of novel therapeutics for human use, provided that the technology of chimeric avian/human fusions could be fully exploited. One should also keep in mind that recombinant technologies can lead to the generation of monoclonal IgY or IgY-like antibodies circumventing the need for animal immunization[72,83], which is desirable from an ethical point of view concerning the animal welfare.

It is important to remind that the IgY technology was introduced in 1990's as an alternative that could at least partly fulfil the ethics requirements set by the 3Rs principle[1,3]. Recently, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) has recommended that “animals should not be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications any longer”, taking into account the Opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) on the scientific validity of replacements for animal-derived antibodies[128]. As referred to by the ESAC, the 2018 Nobel Prize in Chemistry was awarded “for the phage display of peptides and antibodies”[129,130], which, according to the Committee, proves maturity and supports wide application and full exploitation of the phage display technology in the area of antibody production. The EURL ECVAM recommendation may accelerate transformation/switch of the original IgY technology toward development of monoclonal IgYs through phage display techniques that totally avoid the animal immunization step. Total avoidance of animal immunization will further minimize the risk of zoonotic diseases, which is very low but still present when antibodies are produced in chickens, both wild and transgenic.

HIGHLIGHTS

The avian polyclonal antibodies/IgYs have unique and highly desirable functional features.

The term “IgY technology” describes the procedure involving immunization of avian species, consequent isolation of the polyclonal IgYs from the “immune” egg yolk (thus avoiding bleeding and animal stress) and application of the IgYs to various areas of medicine and biotechnology.

During the last decades the IgY technology has been widely used, especially as a means for the efficient *in vitro* immunodetection of biomolecules in many fields of research and disease diagnosis.

Despite the very promising relevant new approaches, there is still space for further exploiting the original IgY technology, due to specific functional, practical and ethical reasons and/or advantages.

CONCLUSION

Until now, development of polyclonal IgYs through the IgY technology has been widely used as a low cost and highly efficient tool, offering a lot of advantages and thus gaining wide application mainly in the *in vitro* immunodetection of biomolecules in biological specimens. Since polyclonal antibodies exhibit some unique functional qualities[131], there is still space for performing research to improve different aspects of the IgY technology. On the other hand, the original IgY technology may “merge” with relevant highly promising approaches, eventually leading, *e.g.*, to worldwide application of non-animal-derived recombinant IgYs or IgY-like immunoglobulins, which, among other benefits, will fulfil strict ethical requirements concerning animal welfare (Figure 1). However, until the practical problems associated with the above-mentioned approaches, *e.g.*, high-cost and/or limited availability of necessary reagents and protocols, have been fully addressed, the original IgY technology still remains a feasible, well-established procedure, in particular for low- and middle-income countries and research laboratories and especially in the field of *in vitro* immunodetection of biomolecules.

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