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# Extracorporeal Circuit Device for Camel Antibodies Production from Blood using Magnetic Nanoparticles

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**The present work describesthe use of nanotechnology in the direct purification of antibodies from life camels.The new method relies on the use ofa simple extracorporeal device coupled with affinity purification to antigen functionalized magnetic nanoparticles that is applied continuously in extracorporeal circuit. The device was connected to camel blood stream by HLS cannulaeveno-venous to allow the blood stream path through the device with the flow rate of ~ 10 ml/min. The new method requires no sacrificing the animal for the recovery of antibodies. Antigen functionalized magnetic nanoparticles are injected into a blood flow where they capture the targeted antibodies by affinity binding. Subsequent removal of the antibody-loaded nanomagnets from the bloodstream takes place by continuous magnetic separation allowing recirculation of the blood for continuous production of more antibodies. For the demonstration of the possibleuse of this new method in purifying the antibodies from immunized camels, lysozyme was used as the antigen for immunization and magnetic nanoparticles functionalization. Immunization of camels was performed fora period of eight-weekwith gradual increase of antigen amount up to 5 or 1 mg antigen. The device was validated via Sandwich ELISA and results indicated the efficacy of the new device in the continuous recovery of camelid antibodies without the need to sacrifice the animal and the need to use antigen at no more than 1 mg for successful antibodies production using the new device at a large scale.**

**Keywords:** *Camelid, Nanomagnet, recirculation, lysozyme.*

Hetero-tetrameric antibodies of mammalian immunoglobulin (IgG) comprise two domains of heavy (H) and two domains of light (L) identical extremely conserved sequence of amino acids in the resulted polypeptide chains (Padlan, 1994). This immunoglobulin structure is called IgG1. Sera of *Camelidae* comprise two additional types of IgG antibodies namely IgG2 and IgG3. These camelid antibodies lack the L chain polypeptide domains and is made of three, not four, domains as they do not contain the first constant domain (CH1). The homo-dimeric proteinof the H chain harbors the variable domain VHH at the N terminal(Muyldermans *et al*., 2009), which is adapted to function in antigen binding due to thelack of variable light chain domain (VL) (Muyldermans*et al*., 1994; Vu *et al*., 1997;Harmsen *et al*., 2001;Maass *et al*., 2007). VHH is known to contain an additional disulfide bond (Cys-Cys) (Conrath *et al*., 2003) that participates in making the loop structure (Muyldermans *et al*.,2001;Genst *et al*., 2006). Overall structure of the camelid IgGas compared to clasicalIgGis shown in *Figure 1*.

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Magnetic nanoparticles were used in various medical, diagnostic and industrial applications (Masashige, 2002). Coupling of the proteinaceous sources, such as antigen, at the surface of magnetic nanoparticles was achieved by several methods using surface activating material and surface functionalized magnetic particles (Pankhurst *et al*., 2003). By the use of this new approach, blood purification system includes filtering antibodies rather than filtering cut-offs or column surface saturation.

The present studydescribesa magnetic separation-based antibody purification systemfrom camel blood using ultra-strong magnetic nanoparticles to be attached to an extracorporeal blood purification circuitfor continuous production of the camelid antibodies without the need to sacrifice the animal.

## MATERIALS AND METHODS

### Camel immunization

Three premature male camels were utilized in the present study. The first was continuously injected with 0.1, 0.5, 1,then 5 mg lysozyme (antigen), while the second was injected with 0.1, 0.2, 0.5, then 1 mg lysozyme. Antigen dose was increased biweekly across the eight-week experiment period. The third animal was injected with wash buffer (negative control)(cat. no. K0001.C.100) of the Lysozyme ELISA kit(Eagle Biosciences, Inc.).Blood samples were taken after eight weeks in order to detect the dynamics of utilizing this device across two concentrations of antigen.

### Antigen immobilization of magnetic nanoparticles

Coupling and blocking of magnetic nanoparticles and protein complex were done using Pierce™ NHS-activated magnetic nanoparticles (Thermo Scientific) following the manufacturer's manual. Magnetic nanoparticles were first washed with buffer A, then 5 mg of NHS-activated magnetic nanoparticles were coupled with10 mg protein (antigen), then,200 ul protein solution mixed with washed magnetic nanoparticleswere incubated at RT for 6 hours, then, washed with wash buffer B and water. The reaction was terminated by washingwith quenching buffer several timesand antigen immobilized nonoparticles were reconstituted in 1 ml PBS buffer and stored at 4ºC until further usage.

### Device construction and purification of camel's antibodies

Antigen functionalized magnetic nanoparticles were dispersed in 300 ml hydroxyl ethyl starch 6%, then added to magnetic-particles-supply reservoir. Then, the device was attached to the immunized animals (10 ml/minflow rate). The device was constructed from the cannula injected

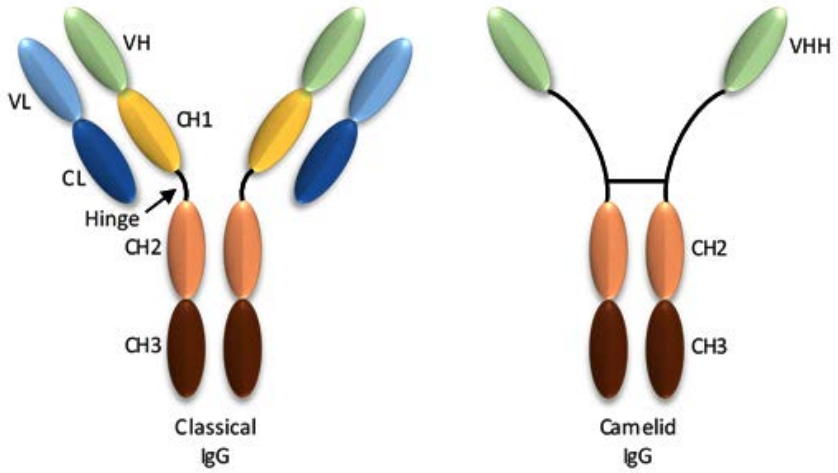


**Fig. 1.** Structure of camelid IgG as compared with the classical IgG. IgG = immunoglobulin, CH = constant heavy chain domain, VH = variable heavy chain domain, CL = constant light chain domain, VL = constant light chain domain, VHH = variable domain of the heavy chain homodimer

to venous allowing blood stream to flow inside the tubes and the antigen functionalized magnetic particles solution to be mixed with camel's blood. Then, nanomagnetswith antigen-antibody complex were pumped out to the magnetic separator tube for nanoparticles removal to allow clean blood to get back to the venous.Collected magnetic nanoparticles were washed in PBS (pH 7.0) three times to remove magnetic nanoparticles, then, antibodies (Abs) were eluted using 0.1 glycine-HCL (pH 2.3) buffer and dialyzed five times against PBS (pH 7.0) buffer. Protein (e.g., antibodies) concentration was determined using the standard BCA protein assay kit (Pierce, IL, USA).

**Sandwich elisa**

Lysozyme camelid antibodies were detected using theLysozyme Sandwich Enzyme-Linked Immunosorbent (ELISA) kit (Eagle Biosciences, Inc.). The assay was done following user's manual except for the use of the ELISA microtiter PVC plates coated with the newly recovered camelidcapture Abs (10 ìg/ml) in carbonate/bicarbonate buffer (pH 9.6)instead of the use of plates pre-coated with anti-human lysozyme capture Abs supplied with the kit. Plates were covered with adhesive and incubated at 4°C overnight. Then, coating solution was cleared, and plates were washed twice by filling wells with PBS (200 ìl). Then, the plates were left to dry. The uncoated protein-binding sites were blocked in the coated wells by the use of 200 ìl blocking buffer(5% non-fat dry milk/PBS/well) overnight then buffer was removed and again plates were left to dry for subsequent use.

Sandwich ELISA included the device-recovered Abs (200 ul at 10 ug/ml) used as the capture Abs,lysozyme (originally used for generating the camelid Abs), used as the antigen, and the indicator system of horseradish peroxidase (HRP)-conjugated rabbit used as the secondary detection anti-lysozyme Abs. A standard curve was prepared byplotting lysozyme antigen at known concentrations against the obtained readout at optical density 450 nm using 800™ TS Absorbance Readers. The Linear plot model was used to present the concentration of lysozyme in one axis and the readout in the other axis. R2 values are used to determine fitting, with values higher than 0.99. Concentration of the antigen was detected from the standard curve as indicated in the user manual. Experiment was done in three replicates and statistically analyzed using paired comparison

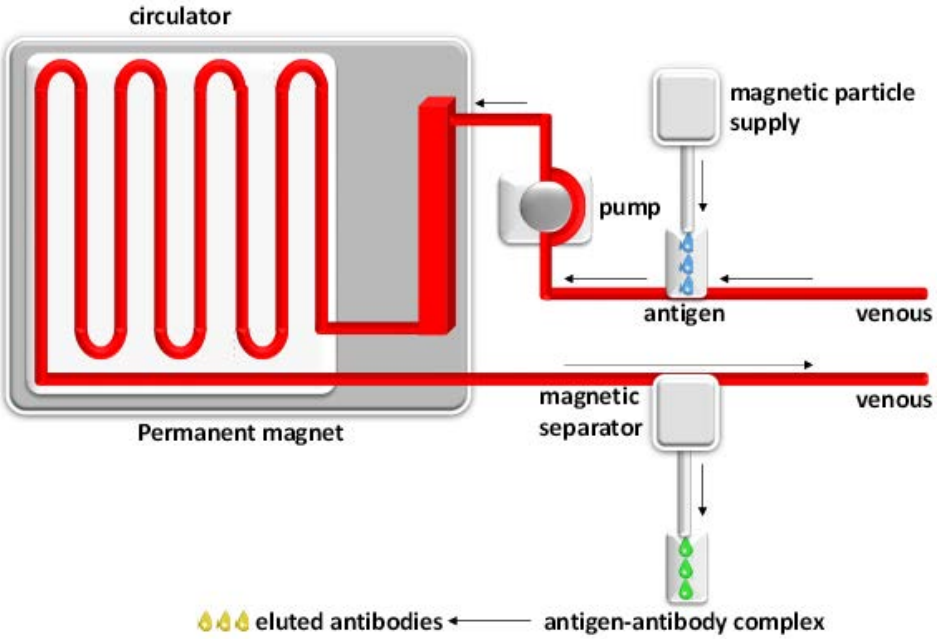


**Fig. 2.** Schematic representation of the extracorporeal circuit device used for continuous production of camelid antibodies

of t test at a = 0.05 for the two antigen-injected animals.

## RESULTS AND DISCUSSION

Pierce™ N-hydroxysuccinimide (NHS)-activated magnetic nanoparticles (Thermo Scientific) were used to covalently immobilize (functionalize) lysozyme protein for the affinity purification of camelid antibodies. The activated magnetic nanoparticles contain NHS functional groups reacting with primary amines on the protein in order to construct a stable amide linkage. Coupling reaction was performed in amine-free buffer (pH 7-9) and nanoparticles were removed from the reaction using a magnetic stand.

As shown in *Figure 2*, the work presents a new device for real-time purification of specific antibody from large animal to increase productivity without the need to sacrifice it. The figure indicates antigen functionalized magnetic nanoparticles that are injected continuously into blood flow where the requiredAbs are specifically captured by affinity binding. Removal of the antibody-loaded nanomagnets from bloodstream takes place by magnetic separation allowing recirculation of the blood for continuous production of more Abs. Cleaning bloodstream from the Absavoids the occurrence of blood coagulation and allows the continuous supply of Abs from alive animal without the need to sacrifice it.

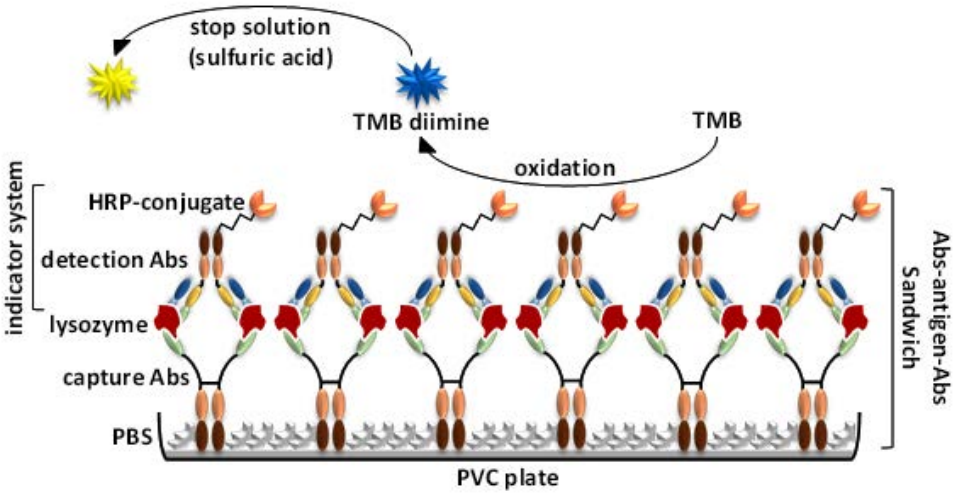


**Fig. 3.** Sandwich ELISAsystem for the detection of lysozyme bound to the newly recovered camelid Ab (capture Abs) in one or more epitopes and to the HRP-conjugated rabbit Abs (detection Abs) in other epitopes

**Table 1.** Lysozyme concentrations calculated from linear logarithmic abscissa for three camels based on the intensity of the yellow color of substrate (TMB) resulted from the HRP enzyme conjugated to secondary Abs of sandwich ELISA "capture Abs-lysozyme-detection Abs". Original amounts of lysozyme at week 8 for the three camels were 5, 1 and 0 (control) mg.

| Camel | Original lysozyme injected (mg) | Calculated lysozyme conc. (ng/ml) |
|---|---|---|
| 1 | 5 | 635.41 |
| 2 | 1 | 547.36 |
| 3 | 0 | 0.79 |

Sandwich ELISA is a sensitive and robust assay that was used in the present study in order to detect the quality and quantity of the recovered camelid Abs using the new wearable device. During this enzyme assay, antigen is sandwiched between two layers of antibodies, namely capture and detection Abs (*Figure 3*).

The assay basically detects presence and quantity of antigen between these two layers of Abs. Capture Abs represent the new camelid Abs, while the detection Abs represent the manufacturer's Abs. The latter is part of the indicator system of Abs with HRP-conjugate that directly indicates the intensity of the antigen (lysozyme) by which detection Abs

bind to, while indirectly report the intensity and affinity of the capture camelid Abs, the focus of the present study. It was expected that camelid Abs will react with one or more epitopes on the antigen that are completely different from the epitopes on the same antigen that react with the rabbit Abs. This speculation was based on the difference in structure of the two types of Abs shown in *Figure 1*.

Sandwich ELISA can be a direct or indirect method of detection. In direct method, antigen can be detected by both capture and detection Abs, while indirect method, as in the present case, secondary Abs are used to detect antigen immobilized by capture Abs. As monoclonal capture Abs recognize a single epitope, polyclonal capture Abs often bind to several epitopes, thus pull down as much antigen as possible. Accordingly, a binding curve is constructed based on the affinity of the detection Absthat directly reflects antigen concentration and indirectly reflects capture Abs concentration and affinity.

The first incubation step of sandwich ELISA in the current study involved binding of lysozyme to the capture camelid Abs coated with PBS on the PVC plate. Unbound antigen was removed by washing to allow secondary Abs linked to HRP enzyme to further bind only to the antigen captured by camelid Abs to form a sandwich of "capture Abs-lysozyme-detection Abs". By doing this, antigen served as a bridge between both types of Abs. The secondary Abs are conjugated with peroxidase that constitutes the indicator system. Then, addition of the colorless tetramethylbenzidine or TMB, a peroxidase substrate, resulted in the production of blue-colored TMB diimine with intensity respective to proportion to the amount of analyte (antigen) present. After adding the acidic stop solution (sulfuric acid), enzymatic reaction is terminated and color changes from blue to yellow (*Figure 3*). The intensity of the yellow color isindirectly proportional to concentration of antigen, e.g., lysozyme, thus, intensity of capture Abs. TMB is a hydrogen donor used for hydrogen peroxidereduction by HRPto water. The recovereddiiminegives the solution a blue color, and the intensity ofblue color is read on a spectrophotometer at the wavelengths of 370 nm. The reaction is halted by addition of acid (sulfuric acid) as a stop reagent to turn TMB's color from blue to yellow at 450 nm (Martin *et al*., 1984).

The results of lysozyme concentrations across time for the three camels are shown in *Table 1*. The results of calculated lysozyme concentration indicated that the amount of 1 mg lysozyme is not significantly different from that of 5 mg (P=0.253). This data indicates that 1 mg lysozyme is sufficient to recover appropriate amounts of the camelid Abs and no higher concentrationof the Abs is required. The reason for considering the least concentration of the antigen is to avoid the chance of blood coagulation when using high concentration across time. The overall results indicated that the new device is effective in recovering camelid Abs from alive camels without the need to sacrifice it.

New technical approaches into dangerous diseases (ex., autoimmune disorders) towards the identification of new therapeutic targets requires the use of new blood purification techniques (Bellomo *et al*., 2001; Tetta *et al*., 2003; Zang *et al*., 2014; Liu *et al*., 2017). Regardless of the current blood purification systems, target-specific filtering of multiple compounds is still a major task, particularly for molecules differing in physicochemical character with high molecular weight compounds (e.g., proteins). As diffusion is slow and filtration is inappropriate due to filter cut-offs, patients requiringexchange of centrifugal plasma or blood purification through adsorbents. Nonetheless, antibody-coated adsorbents (Du Moulin *et al*., 1993) or microsphere-based detoxification systems (MDS) (von Appen *et al*., 1996; Weber *et al*., 2001) are currently being introduced to specifically aim at therapeutic targets. Spherical nonporous particles used as adsorbents was shown to take advantage from the intrinsically better surface accessibility due to the higher external surface areas and the shorter diffusion distances as compared to membranes (Herrmann *et al*., 2009). Herrmann *et al*. (2010) described the appraoch of magnetic blood purification system using target-specific metal nanomagnets to get red of toxins from blood more efficiently. The latter approach of toxin purification is analogue to that adopted in of the present work.

Through the presentwork, thedeveloped small device can be easily attached externally in the camel's body in order to extract specific antibodies from circulating blood without the need to sacrifice the animal.This device provides a simple, safe, cost-effective and time-saving

method for appropriate antibodies production and purificationfrom camels at large scale.

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