Production of *Leptospira* LipL32 Specific-monoclonal Antibodies Conjugated to Dextran-coated Iron Oxide Magnetic Nanoparticles

การผลิตอนุภาคแม่เหล็กเคลือบด้วยสารเดกแทรนที่เชื่อมต่อกับโมโนโคลนาลแอนติบอดีจำเพาะต่อโปรตีนลิบแอลสามสิบสองของเชื้อเลปโตสไปรา

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ABSTRACT

This study aimed to produce anti-LipL32-dextran-coated-iron oxide magnetic nanoparticles using two monoclonal antibodies (mAbLPF1 and mAbLPF2) specific to surface exposed epitope peptides of *Leptospira*, LipL32. Magnetic nanoparticles were synthesized by co-precipitation method and exhibited ferromagnetic property. The particles were almost uniformly distributed, with particle size less than 50 nm under scanning electron microscopy. The magnetic nanoparticles were coupled with anti-LipL32 immunoglobulins by periodate oxidation and Schiff base linkage methods. Percent entrapment of mAbLPF1 and mAb LPF2 to the particles were 16 and 36%, respectively, when compared to BSA control (29.6%). Immunomagnetic separation of recombinant LipL32 protein was performed and the capture efficiency of mAbLPF1-MNP and mAbLPF2-MNP were 60.07% and 34.10%, respectively.

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อผลิตอนุภาคแม่เหล็กเคลือบด้วยสารเดกแทรนที่เชื่อมต่อกับโมโนโคลนาลแอนติบอดีที่จำเพาะต่อโปรตีนลิบแอลสามสิบสองของเชื้อเลปโตสไปรา อนุภาคแม่เหล็กถูกสร้างขึ้นด้วยเทคนิคการตกตะกอนร่วม อนุภาคที่สร้างขึ้นมีขนาดเล็กกว่า 50 นาโนเมตร และมีคุณสมบัติเป็นแม่เหล็ก ผู้วิจัยทำการเชื่อมต่อกับอิมมิวโนโกลบูลินที่มีความจำเพาะต่อโปรตีนลิบแอลสามสิบสองสองตัว คือ โมโนโคลนาลแอนติบอดี LPF1 และ LPF2 บนพอลิเมอร์เดกแทรนของอนุภาคแม่เหล็ก โดยแอนติบอดี LPF1 และ LPF2 เชื่อมต่อกับอนุภาคแม่เหล็กได้ร้อยละ 16 และ 36 ตามลำดับในปริมาณที่เท่ากันเมื่อเปรียบเทียบกับโปรตีน BSA คือ ร้อยละ 29.6 การทดสอบประสิทธิภาพในการจับและแยกโปรตีนลูกผสมลิบแอลสามสิบสองด้วยอนุภาคแม่เหล็กเคลือบด้วยสารเดกแทรนที่เชื่อมต่อกับโมโนโคลนาลแอนติบอดีชนิด LPF1 และ LPF2 ด้วยวิธี immunomagnetic separation พบว่ามีประสิทธิภาพในการจับร้อยละ 60.07 และ 34.10 ตามลำดับ

Key Words: Leptospirosis, Dextran-coated iron magnetic nanoparticles, LipL32 specific-monoclonal antibody

คำสำคัญ: โรคไข้ฉี่หนู อนุภาคเหล็กเคลือบด้วยสารเดรกแทรน โมโนโคลนาลแอนติบอดีจำเพาะต่อโปรตีนลิบแอลสามสิบสอง

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Introduction

Human leptospirosis, a zoonotic disease has been recognized as a global public health problem worldwide as well as in northeast region of Thailand. The disease is caused by an infection of spirochaetal bacteria of the genus *Leptospira* by direct or indirect contact with contaminated water and soil or infected animals, such as rodents, dogs and cows, including their waste especially urine (Adler and de la Pena Moctezuma, 2010; Thipmontree *et al*., 2014). Symptoms of leptospirosis are similar to other febrile illnesses, for example malaria, dengue and rickettsia infections. Additionally, due to rapid fatality rate of leptospirosis the antibiotics should be provided as soon as leptospirosis is suspected. As a consequence, early and accurate laboratory testing is crucial for a proper differential diagnosis and treatment of leptospirosis. A new method with improved sensitivity and easily operated would be beneficial for diagnosis of leptospirosis. Members of the genus *Leptospira* are traditionally classified into two groups, i.e., *Leptospira interrogans* which all are pathogenic and *L. biflexa* which are non-pathogenic, free living saprophytes. Within the genus, there are more than 300 different antigenic serovars.

LipL32, a dominant outer membrane lipoprotein (approximately 38,000 copies per cell) with highly conserved in both pathogenic and intermediately pathogenicity clades leptospires, has been used as vaccine candidate and *lipl*32 DNA molecular target for diagnosis of acute leptospirosis (Umthong *et al*., 2014; Humphryes *et al*., 2014 and Monte *et al*., 2012). LipL32 was also used as diagnostic target, i.e., LipL32-ELISAs, immunochromatographic test (Chalayon *et al*., 2011; Chirathaworn *et al*., 2013). Anti-LipL32 mAbs have been used as reagent in immunodiagnosis for leptospirosis (Coutinho *et al*., 2007) as well as in immuno-magnetic diagnosis approaches for capturing leptospires. (Fernandes *et al*., 2008; Monte *et al*., 2012). Two hybridoma secreting monoclonal antibodies (MAbs), mAbLPF1 and mAbLPF2 specific to surface exposed epitope peptides of *Leptospira* LipL32 were established (Maneewatch *et al*., 2008; 2014).

In this study, we coupled anti-LipL32 mAbs, mAbLPF1 and mAbLPF2, with dextran-coated iron oxide magnetic nanoparticles and the prepared anti-LipL32-dextran-MNPs were used as a material for capturing and separating recombinant LipL32 protein.

Objective of the study

In this study, we aimed to produce monoclonal antibodies (*Leptospira* LipL32-specific mAbs) conjugated with dextran-coated iron oxide nanoparticles for using as a material for capturing and separating LipL32 antigen and intact leptospires from several sample sources.

Methodology

Chemicals

Ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), sodium periodate ($NaIO_4$), sodium sodium borohydride ($NaBH_4$) and ammonium hydroxide (25 wt%) were purchased from Merck (Darmstadt, Germany). Dextran sulfate (5, 50, and 500 kDa) was purchased from MP Biomedicals, LLC (France). All other chemicals were of analytical reagent grade and were used without further purification.

Mouse Monoclonal antibodies

Mouse monoclonal antibodies (MAbs) specific to LipL32, mAbLPF1 and mAbLPF2 (Maneewatch *et al*., 2008) and mAb204D3 specific to LPS of group D *Salmonella* Typhi (Chaicumpa *et al*., 1988) were kindly provided by Prof. Dr. Wanpen Chaicumpa, Mahidol University. The monoclonal immunoglobulins (Igs) were individually prepared in protein-free hybridoma medium, subsequently concentrated and buffer exchanged to phosphate buffered saline (PBS), pH 7.4 using Amicon Ultra-tubes (Millipore, Billerica, MA). Concentration of the monoclonal Ig was determined at OD_{280nm} and kept at -20 ºC until use. Antigenic specificity of anti-LipL32 was verified by Western blot analysis. (Maneewatch *et al*., 2014)

Recombinant LipL32 protein

LipL32 protein was produced from a transformed BL21 (DE3) *Escherichia coli* containing *lipl32*-pET23a plasmid as previously described (Maneewatch *et al*., 2008). The recombinant LipL32 was purified by using immobilized metal affinity column (Ni-NTA resin, GE Healthcare, Sweden) by AKTA purifier system (GE Healthcare, Sweden). Purified LipL32 protein was verified for purity and antigenic specificity by SDS-PAGE and Coomassie Brilliant Blue G-250 staining and Western blotting using anti-6x His tagged antibody, respectively. Protein concentration was determined by Bradford assay (Bradford, 1976) using Bio-Rad Protein Assay (Bio-Rad Laboratories, USA) at wavelength of 595 nm.

Synthesis of dextran-coated iron oxide nanoparticles

Dextran-coated iron oxide nanoparticles were synthesized by a co-precipitation method with modification (Martin *et al*., 2004). Briefly, ferric chloride hexahydrate ($FeCl_3.6H_2O$) and ferrous chloride tetrahydrate ($FeCl_2.4H_2O$) in 1:2 molar ratio were dissolved in deionized water and mixed with 0.15 M dextran sulfate. The mixture was then heated to 70 ºC under constant nitrogen gas in a three-necked flask. Thereafter, 25% ammonium hydroxide (NH_4OH) was added quickly into the solution under vigorous stirring until required pH was reached and then the solution was heated to the required time. After heating, the colloidal dextran-iron particles were separated by magnetic stand and the particles were washed several times with deionized water, and finally were resuspended in PBS pH 7.4 The particles' size was measured by scanning electron microscope (SEM, Hitachi S-570; Hitachi High-Technologies Co., Ltd, Japan).

Conjugation of immunoglobulin to dextran-coated iron oxide nanoparticles

Surface hydroxyl of dextran-coated iron oxide nanoparticles were oxidized by adding 3 mg/mL of $NaIO_4$ and incubated for 40 minutes at room temperature in the dark. Excessive periodate was removed by the magnetic stand and the particles were washed several times with 25 mM Tris-HCl containing 0.15 M NaCl at pH 8.0. In this step, bovine serum albumin (BSA) was used to adjust the conjugation. BSA in various concentrations (5, 10, 20, 50, 75, 100, 125, 150, 200 µg/mL) was added into the iron oxide particles. Thereafter, the generated Schiff bases were stabilized by reductive amination (Murray *et al*., 1989) in the presence of $NaBH_4$ at a final concentration of 1 mg/mL and then were then incubated overnight at 4 ºC. The MAbs conjugated dextran-coated iron oxide nanoparticles were subsequently precipitated by placing on magnetic stand. The supernatant was removed and were washed several times with 25 mM

Tris-HCl containing 0.15 M NaCl at pH 8.0. Anti-LipL32-dextran-iron-nanoparticles were resuspended in PBS, pH 7.4 and stored at 4 °C.

Immunomagnetic separation

The conjugated nanoparticles were tested by mixing with 225 µg of LipL32. The incubation of LipL32 with particles was performed at RT for 30 minutes. A similar protocol was performed with the BSA-coated particles. After incubation, the tubes were placed on the magnetic stand for the separation particles from unbound LipL32. After magnetic separation, LipL32 was eluted from particles by adding 10% SDS and then was boiled at 95°C for 10 minutes. The amount of LipL32 that can entrap onto the particles was determined by ELISA method.

LipL32 ELISA

LipL32 protein (100 µl) was immobilized onto the 96-wells microtiter plate and was air-dried at 37 ° C. Plates were then blocked with blocking reagent [1% BSA in PBS plus 0.5% Tween 20 (PBST)] at 37 ° C for 30 minutes. After washing three times with PBST, LipL32-specific Ab (100 µl) were added into each wells and incubated at 37° C for 30 minutes. Unbound antibody was removed and the plate was washed with PBST. Horseradish peroxidase-conjugated goat anti-mouse IgG (Dakopatts, Glostrup, Denmark) (1:10,000 dilution) was added into each well. The TMB enzyme substrate was added into wells (100 µl per well). The reaction was allowed to take place in the dark for 30 minutes and then was stopped by adding 50 µl of 0.4 M H_2SO_4. The optical density was measured at 450 nm against reagent blank control by an ELISA reader (Sunrise™; TECAN, Switzerland). Amount of LipL32 was relatively quantified from standard LipL32 curve.

Results and Discussion

A magnetic iron oxide nanoparticle with cross-linked dextran coating is a powerful and illustrative nanoparticles employed in various molecular applications such as imaging, diagnosis and therapy (Tassa *et al*., 2011). Immuno-magnetic separation is one of the pre-concentration techniques which is commonly used for sample preparation and cleanup prior to detection and have been applied for several purposes (Tangchaikeeree *et al*., 2013; Yang *et al*., 2014) as well as sensitivity improvement of leptospirosis diagnosis (Monte *et al*., 2012; Fernandes *et al*., 2008; Yan *et al*., 1998). In this study, we attempted to develop dextran coated magnetic nanoparticles conjugated with monoclonal antibodies specific to *Leptospira* LipL32.

Firstly, dextran-coated iron oxide nanoparticles were synthesized by a co-precipitation method. Three different dextrans (molecular weight of 5, 50 and 500 kDa) and amount of iron particles were optimized in order to produce dextran coated iron oxide MNPs with highest entrapment efficiency. In this experiment, BSA was used as representative protein in conjugation reaction with the synthesized particles. As shown in Fig. 1, 10 mg of magnetic nanoparticles and 50 and 500 kDa dextran showed highest entrapment efficiency. Magnetic nanoparticles (10 mg) and 50 kDa dextran were chosen for preparing the dextran-coated iron oxide nanoparticles.

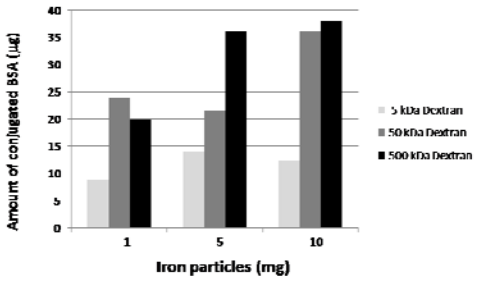

Figure 1 Optimization of dextran (5, 50, 500 kDa) and iron particles (1, 5, 10 mg) for preparing dextran-coated iron oxide nanoparticles. The amount of BSA conjugated onto the synthesized particles with varied molecular weights of dextran and amount of magnetic nanoparticles were determined.

The synthesized particles demonstrated ferromagnetic property (data not shown). Morphology and size of the dextran-ferromagnetic particles were assessed by scanning electron microscope as shown in Fig. 2. It was observed that particles were almost uniform and particle size was less than 50 nm.

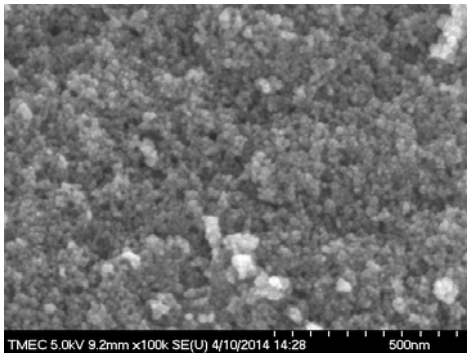

Figure 2 SEM micrograph of the synthesized dextran-coated iron oxide nanoparticles. The image was recorded on Hitachi S-570.

In order to optimize amount of immunoglobulin and conditions for immunoglobulin coupled with oxidized dextran-coated iron oxide nanoparticles reaction, BSA was used as control in the conjugation reaction. Appropriate amount of protein required to couple onto the dextran-coated naoparticles was determined. The amount of bound BSA was determined from the depletion of protein after conjugation process. It was observed that the highest amount of BSA that could bind onto the dextran-coated magnetic nanoparticles was approximately 57 µg when total of 125 µg of BSA were used in the reaction (Fig. 3).

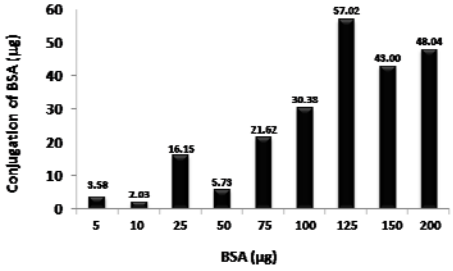

Figure 3 Total BSA conjugated onto MNPs with varied concentrations. The depletion of BSA after the conjugation process was calculated and plotted against the total amount of BSA conjugation.

Incubation time and temperature for conjugation process were also optimized. Conjugation of BSA onto dextran-coated iron oxide nanoparticles was performed at three different temperatures, 4, 25 and 37 °C, with different incubation times, 1, 2 and 4 hours, (Table 1). The suitable condition for incubation was incubated the magnetic nanoparticles with BSA for 4 hours at 4 °C and this condition was used in the next step.

Table 1 Optimization of incubation times and temperatures in conjugation reaction. BSA (125 µg) protein was conjugated onto dextran-coated MNPs with varied incubation times (1, 2 and 4 hours) and temperatures (4, 25 and 37 °C). Percent entrapment efficiency of BSA coupled with MNPs was determined from bound BSA.

BSA to dextran-coated MNPs		Entrapment efficiency of BSA to oxidized dextran-iron-MNPs (%)
Temperature (°C)	Time (hours)	
4	1	55.2
	2	56.0
	4	56.8
25	2	53.6
37	2	49.6

It has been reported that anti-LipL32 mAbs could bind to native LipL32 on live, intact spirochetes (Fernandes *et al*., 2007) and anti-LipL32 mAbs have been used as reagent in immuno-magnetic diagnosis approach for capturing leptospires. (Monte *et al*., 2012; Fernandes *et al*., 2008). In this research, two monoclonal antibodies, mAbLFP1 and mAbLFP2, which are specific to different surface-exposed epitopes peptides of LipL32 (Table 2; Maneewatch *et al*., 2008; 2014) were prepared and their specificity were verified by Western blotting. Control mAb204D3 did not react to recombinant LipL32 protein (data not shown). In order to prepare anti-LipL32 coupled with dextran-iron-MNPs, equally amount (30 µg) of mAbLFP1 and mAbLFP2 immunoglobulins were conjugated onto oxidized dextran-coated iron oxide MNPs in the selected condition (4 hours of incubation at 4 ° C). Bound immunoglobulins were determined by Bradford assay. Both mAbs could conjugate onto oxidized dextran-iron-MNPs (Table 2). Preliminary result of entrapment efficiency of the mAbLPF1 and mAbLPF2 to dextran-iron MNPs were 16% and 36%, respectively when compared to BSA control (29.6%).

Capture efficiency of anti-LipL32-dextran-iron-MNPs with recombinant LipL32 was performed by immunomagnetic separation method. Amount of bound LipL32 protein was determined by LipL32-ELISA assay and relatively quantified by using LipL32 standard curve. It was found that LipL32 protein could be captured and separated by anti-LipL32 coupled dextran-iron-MNPs from the mixture.

Table 2 Entrapment efficiency of anti-LipL32 immunoglobulins to dextran-iron-MNPs and capture efficiency of anti-LipL32-dextran-iron-MNPs with recombinant LipL32 protein.

Magnetic nanoparticles	Specificity[1]	Percent immunoglobulin entrapment[2] (%)	Percent LipL32 capture[3] (%)
mAbLPF1-dextran-iron-MNP	*Leptospira* LipL32	16	60.07
mAbLPF2-dextran-iron-MNP	*Leptospira* LipL32	36	34.10
BSA-dextran-iron-MNP	Nd	29.6	undetectable

[1] Epitope peptides of LPF1 and LPF2 mAbs were located at carboxy and middle domains of LipL32 protein (Maneewatch *et al*., 2014).

[2] Dextran-iron-MNPs were coupled with 30 µg of individual mAbLPF1, mAbLPF2, and BSA. Protein concentrations were determined by Bradford assay.

[3] LipL32 protein (100 µg) was captured by anti-LipL32-dextran-MNPs by immunomagnetic separation and bound LipL32 was determined by LipL32-ELISA.

Nd, not determined

Conclusion

In summary, we have successfully synthesized anti-LipL32-dextran-iron oxide magnetic nanoparticles with ferromagnetic, and LipL32-captured properties. The nanoparticle will be more characterized and developed as a material for capturing and separating LipL32 antigen from lysed leptospires as well as live spirochete from clinical specimens in order to improve the diagnostic sensitivity.

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