Final Report

Project Title

Rapid detection of mutans streptococci

by substrate specific binding of automutanolysin

By

Panida Thanyasrisung

September 2016

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Rapid detection of mutans streptococci

by substrate specific binding of automutanolysin

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ชื่อโครงการ :   การตรวจหาเชื้อมิวแทนส์ สเตรปโตคอคไคอย่างรวดเร็ว โดยอาศัยความจำเพาะต่อการจับซับสเตรตของเอนไซม์ออโตมิวตะโนไลซิน

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ระยะเวลาโครงการ: 2 ปี

การตรวจเชื้อมิวแทนส์ สเตรปโตคอคไคอย่างรวดเร็วข้างเก้าอี้เป็นตัวช่วยที่สำคัญในการประเมินความเสี่ยงของการเกิดโรคฟันผุ ชุดตรวจเชื้อสเตรปโตคอคคัส มิวแทนส์สำเร็จรูปสามารถหาซื้อได้ในท้องตลาด แต่ก็ยังมีน้อย ออโตมิวตะโนไลซิน (เอเอ็มแอล) เป็นเอนไซม์ในกลุ่มเปปทิโดไกลเคน ไฮโดรเลส ซึ่งส่วนของโดเมนที่ทำหน้าที่จับกับผนังเซลล์ของเอนไซม์นี้มีความจำเพาะในการจับกับเชื้อกลุ่มมิวแทนส์ สเตรปโตคอคไค การศึกษานี้จึงมีวัตถุประสงค์ที่จะพัฒนาวิธีการตรวจอย่างรวดเร็วโดยใช้โดเมนที่ทำหน้าที่จับกับผนังเซลล์มาเชื่อมกับเอนไซม์ฮอร์สเรดิชเปอร์ออกซิเดส อย่างไรก็ตามโปรตีนลูกผสมที่ได้อยู่ในรูปที่ไม่ละลายน้ำ ดังนั้นอนุภาคแม่เหล็กนาโนจึงถูกนำมาใช้เป็นทางเลือกของตัวแสดงผลในการยึดติดกับโดเมนที่ทำหน้าที่จับกับผนังเซลล์ (CWBD-conjugated MNPs) โดยอนุภาคแม่เหล็กนาโนนี้จะตรึงด้วยพอลิอะคริลิกแอซิดผ่านพันธะเอไมด์เพื่อให้มีหมู่คาร์บอกซิลไว้ใช้สำหรับยึดกับโดเมนที่ทำหน้าที่จับกับผนังเซลล์ของเอนไซม์เอเอ็มแอล เพื่อตรวจสอบความสามารถในการจับ และความจำเพาะของสารประกอบอนุภาคนี้ต่อเชื้อกลุ่มมิวแทนส์ สเตรปโตคอคไค และเชื้อสเตรปโตคอคคัส แซงกิวนิส และเชื้อสเตรปโตคอคคัส ซาลิวาเรียส สารประกอบอนุภาคนี้ถูกผสมรวมกับเชื้อแต่ละตัวที่อยู่ในอาหารเลี้ยงเชื้อ และหลังจากนั้นทำการแยกประกอบอนุภาคนี้ออกมาจากอาหารเลี้ยงเชื้อโดยอาศัยสนามแม่เหล็กภายนอก เมื่อแยกออกมาแล้วสารประกอบอนุภาคจะถูกนำ

แขวนลอยในฟอสเฟตบัฟเฟอร์ซาลีน   สารประกอบอนุภาคที่สามารถจับเชื้อได้จะถูกแยกออก
จากที่จับเชื้อไม่ได้โดยการกรองแบบสูญญากาศผ่านกระดาษกรองขนาด 0.8 ไมโครเมตร ซึ่ง
พบว่ามีความสัมพันธ์ระหว่างความเข้มของจุดสีของอนุภาคแม่เหล็กที่ถูกกรองค้างอยู่บน
กระดาษกรอง กับจำนวนเชื้อกลุ่มมิวแทนส์ สเตรปโตคอคไคที่ช่วง 10^2-10^7 CFU/ml แต่ไม่พบ
ความสัมพันธ์นี้กับเชื้อสเตรปโตคอคไคอีกสองสายพันธุ์   สำหรับการนำไปใช้จริงในทางคลินิก
จะต้องมีการทดสอบประสิทธิภาพในการตรวจวัดเชื้อมิวแทนส์ สเตรปโตคอคไคของสารประกอบ
อนุภาคนี้กับเชื้อผสม รวมถึงในน้ำลายจริงจากผู้ป่วยเพิ่มเติมในอนาคต

คำหลัก : การตรวจอย่างรวดเร็ว, สเตรปโตคอคคัส มิวแทนส์, มิวแทนส์ สเตรปโตคอคไค,
อนุภาคแม่เหล็กนาโน และเปปทิโดไกลเคน ไฮโดรเลส

Abstract

Project Code :   TRG5780043

Project Title :   Rapid detection of mutans streptococci by substrate specific

binding of automutanolysin

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Project Period :   2 years

Chair-side rapid detection of mutans streptococci is an important aid to clinical dental caries risk assessment. Rapid *Streptococcus mutans* detection tools are available on the market but there are a small number. Automutanolysin (Aml) is a peptidoglycan hydrolase whose cell wall-binding domain (CWBD) has substrate-specificity towards mutans streptococci. This study aims to develop a rapid detection assay using CWBD conjugated with horseradish peroxidase (HRP). However, the recombinant protein was an insoluble form. Therefore, magnetic nanoparticles were used as an alternative reporter to conjugate with CWBD (CWBD-conjugated MNPs). Magnetic nanoparticles were grafted with poly(acrylic acid) (PAA) providing active carboxyl groups for conjugation with CWBD of Aml. To determine binding ability and specificity of CWBD-conjugated MNPs against mutans streptococci and *Streptococcus sanguinis* and *Streptococcus salivarius*, the composite particles were mixed with single-species bacterial solution and then isolated with external magnet. The isolated CWBD-conjugated MNPs were then re-suspended in phosphate buffer saline. Bacteria-bound CWBD-conjugated MNPs were separated from unbound one by vacuum filtration through a 0.8 µm nitrocellulose filter membrane. There was a linear relationship

between the intensity of colored spots of filtered-MNPs on a membrane and the number of mutans streptococci (range 10^2-10^7 CFU/ml) but not with other two streptococci. Addition of 3,3',5,5'-Tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) enhance a signal of MNPs and thus improve the detection limit. For clinical application, the detection ability against mutans streptococci in mixed culture and also patient's saliva will be further examined.

Executive Summary

Caries risk assessment (CRA) is a method that evaluates the risk of caries development in an individual. This method allows dental professionals to be more efficient and to provide successful prevention and treatment. CRA assesses an individual's risk based on several factors such as past caries experience, dietary habits, socioeconomic status, and laboratory tests (bacterial caries activity tests, salivary flow rate and buffer capacity). For microbiological caries activity test, bacterial culture is necessary. Since this process requires specific laboratory supplies and time to grow bacteria, it is not practical for rural areas. Currently, a rapid detection kit based on antigen-antibody reaction is available in markets. Gao and colleagues reported that this kit could determine the number of *S. mutans* in saliva samples more accurately than the culture-based method [1]. However, the classification of caries risk is still based on the salivary MS levels obtained from the culture-based method.

Automutanolysin (Aml) is peptidoglycan hydrolase showing substrate specificity towards mutans streptococci (MS) but not to other oral streptococci. The substrate specificity to MS is corresponded to a cell wall binding domain (CWBD) of Aml. With this unique ability, CWBD can be a probe for detection of MS. To visualize the target bacteria, a chromogenic/fluorescent reporter is required. The enzyme horseradish peroxidase (HRP) catalyzes the oxidation of substrates in the presence of hydrogen peroxide, resulting in a colored product. At the beginning, we constructed the recombinant protein between CWBD and HRP but the purified protein was in an insoluble form.

Recently; magnetic nanoparticles (MNPs) became a novel method to isolate/concentrate targeted-bacteria even in a dilute sample containing various background materials due to their reaction to magnetic field. Moreover, MNPs exhibit brown color that can be detected by naked eyes. Therefore, the aim of this study is to develop a semi-quantitative rapid detection assay of mutans streptococci using CWBD-conjugated MNPs.

Magnetic nanoparticles (MNPs) were synthesized by solvothermal method and then grafted with poly(acrylic acid) (PAA). Surface-grafted PAA provide carboxyl groups for conjugation with cell wall binding domain (CWBD) of Aml. For CWBD, his-tagged CWBD proteins were expressed from a recombinant CWBD plasmid in *Escherichia coli* BL-21. The proteins were then purified using HIS-Select[®] Nickel Affinity Gel under native condition. The active fractions were pooled and dialyzed against 0.1M phosphate buffer (pH 6.8). Purifed-CWBD proteins were immobilized on PAA-grafted MNPs by EDC/NHS coupling method. To determine recognition and differentiation abilities of CWBD-conjugated MNPs, mutans streptococci, including *S. mutans* and *S. sobrinus*, and other two oral streptococci (*S. sanguinis* and *S. salivarius*) were used in the study. Each of bacterial culture was prepared in various concentrations in a range of 10^2 to 10^7 CFU/ml. Each culture was then mixed with CWBD-conjugated MNPs and incubated at 4 $^{\circ}$C for 30 min. After incubation, the CWBD-conjugated MNPs were isolate from culture by an external magnet and then re-suspended in 0.1 M phosphate buffer saline (pH 7.4). The suspension was filtered through a 0.8 μm nitrocellulose membrane by a vacuum. Bacteria-bound CWBD-conjugated MNPs were remained on the membrane since their sizes are larger than the size of membrane pores. The color intensity of bacteria-bound CWBD-conjugated MNPs spots on a membrane were quantified using a

scion image. We found that mutans streptococci exhibits higher color intensity than the other two oral streptococci. Moreover, the linear regression analysis showed the strong relationship between the colored intensity and the number of bacteria which means the color can used to approximate the number of bacteria. All of these results indicated a potential to develop the CWBD-conjugated MNPs as a chair-side mutans streptococci detection tool for distinguishing the high caries risk group (mutans streptococci >10^6 CFU/ml) from other groups.

Introduction to the research problem and its significance

Dental caries still remains an important oral health problem in many countries. It is caused by multiple factors such as cariogenic bacteria (*S. mutans* and *S. sobrinus*), fermentable sugars, host factors and time. All these factors are different among individuals leading to the difference in caries development. However, the management of dental caries in many countries including Thailand did not focus on individual risk. The high-risk caries patients receive the same treatment and prevention as the low-risk caries group. This may be a reason why the prevalence of dental caries has remained at the similar level for more than 20 years in Thailand [2].

Caries risk assessment (CRA) is a method that evaluates the risk of caries development in an individual. The method allows dental professionals to be more efficient and successful in planning for prevention and treatment. CRA assesses an individual's caries risk based on several factors such as past caries experience, dietary habits, socioeconomic status, saliva characteristics, and the amount of cariogenic bacteria [3-5]. Most factors can be tested at the chair-side except bacterial detection. There are commercial bacterial detection kits that are more convenient and easier to use than conventional ones. However, they still require laboratory supplies, expensive equipment and incubation time that are not practical for working in community dental services [6, 7]. In the past few years, commercial *S. mutans* rapid detection kit based on antigen-antibody reactions became available. The assay used monoclonal antibodies specific for *S. mutans* as a probe. The kit could determine the number of *S. mutans* in saliva samples more accurately than the culture-based method [1]. However, the classification of caries risk is still based on the salivary MS levels obtained from the culture-based method.

Automutanolysin (Aml) is a peptidoglycan hydrolase produced by *S. mutans*. The enzyme selectively digests *S. mutans* and also *S. sobrinus,* which are bacteria causing caries in human[8]. This substrate specificity towards cariogenic bacteria is related to a cell wall binding domain (CWBD) of the enzyme (unpublished observation). So, CWBD has a potential to be a specific probe. Magnetic nanoparticles (MNPs) have been an interesting novel material used for bacterial detection since they, with some modifications, can separate targeted-bacteria from biological samples by using an external magnet and exhibit detectable color[9, 10]. The color can be semi-quantitated by naked eyes, so it is useful for the development of tools to be used in community health services.

Therefore, the objective of this study is to develop a rapid mutans streptococci detection using a conjugation of the cell wall binding domain of Aml and magnetic nanoparticles.

Objectives

1. To develop a probe specific to mutans streptococci for rapid detection assay using a genetically engineered CWBD of Aml linked to HRP.

2. To develop a probe specific to mutans streptococci for rapid detection assay using CWBD of Aml conjugated with PAA-grafted MNPs.

3. To evaluate the specific affinity of CWBD-conjugated MNPs to mutans streptococci.

4. To examine the quantitative ability of the chromogenic assay in the detection of mutans streptococci.

Research methodology

Objective 1) To develop a probe specific to mutans streptococci for rapid detection assay using a genetically engineered CWBD of AmI linked to HRP.

- **Construction of CWBD-HRP fusion gene and expression of CWBD-HRP protein**

Plasmid pET-26b (+) containing HRP gene with a directed evolution (pETpelBHRP1A6Kan, Biotechnol Progress, 1999: 15: 467–471.) was a kind gift of Prof. Frances H. Arnold (Caltech, CA, USA) (Fig.1A). The HRP gene was inserted into the vector as a fusion to the signal peptide pelB enhancing protein solubility. CWBD of AmI with 6xhistidine tag was constructed in pColdIII vector with *capA* gene (Fig.1B). The gene also helps in protein solubility.

A)

B)

pET26b(+)-HRP-2pelB
6313 bp

pColdIII-PT4-6xHis
6640 bp
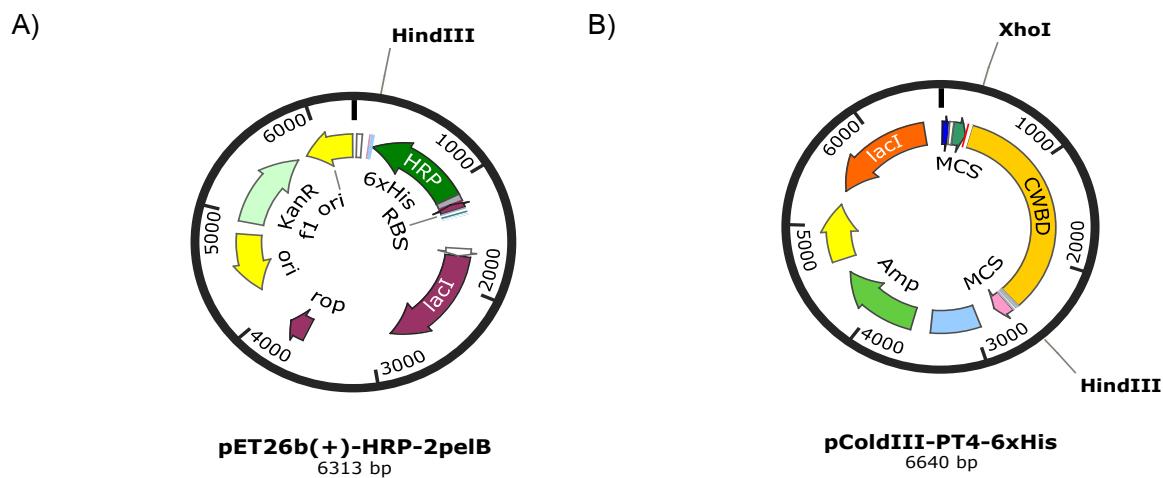
Figure 1 A) HRP gene in pET26b(+) vector and B) CWBD gene with 6xHis in pColdIII

HRP gene with pelB sequence was amplified by PCR with *Nde*I restriction sites and was inserted into pColdIII-CWBD-6xHis plasmid (Fig. 2).
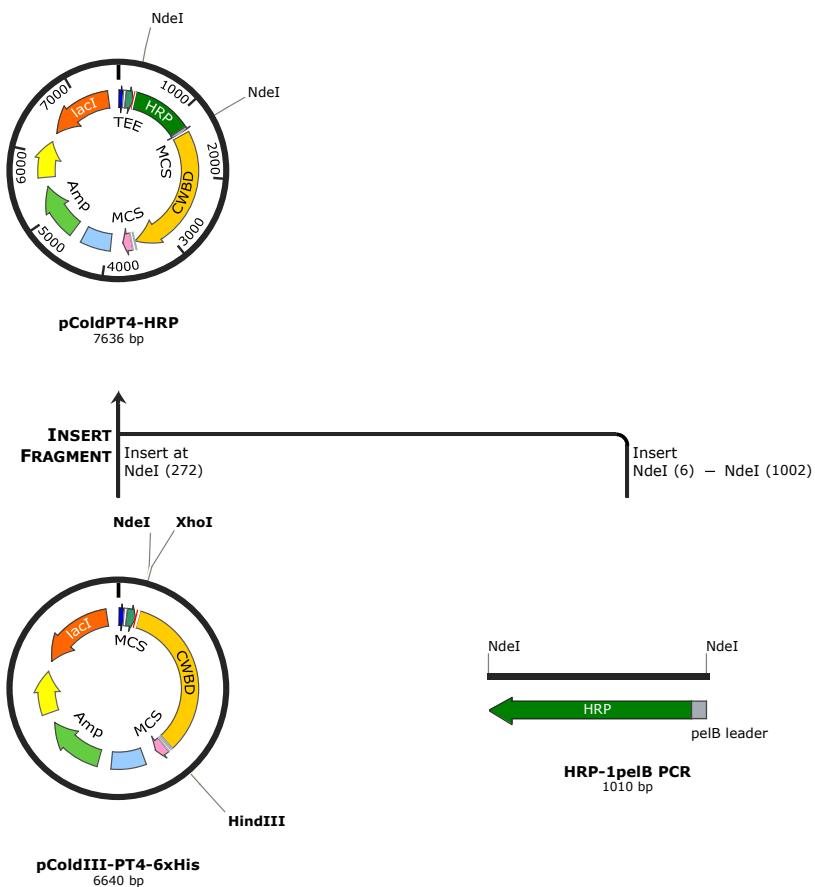
Figure 2 Construction of CWBD-HRP fusion gene in pColdIII vector.

The expression of 6xHis-tagged HRP-CWBD protein (size ~ 120 kDa) was induced by the addition of 1 mM isopropyl-ß-D-thiogalactoside (IPTG) and incubated at 15°C for 24 hours. The bacterial cells were collected and broken by osmotic shock method. The periplasm, cytoplasm and total cell extracts were run SDS-PAGE gel and stained by Coomassie Brilliant Blue to examine the protein solubility. The protein was in cytoplasm that is an insoluble form (Fig. 3).
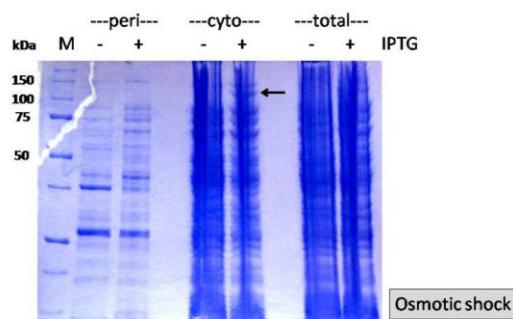
Figure 3 The expression of CWBD-HRP protein (120 kDa)

To improve the protein solubility, we varied IPTG concentration from 0.1-1mM in order to reduce the rate of protein synthesis. We also changed the growth medium by adding 1% glucose to repress induction of the *lac* promoter by lactose which is present in most rich media and adding 1% sorbitol to increase osmotic pressure for accumulation of osmoprotectants to stabilize the native protein structure. Moreover, we changed the vector from pColdIII to pCold ProS2 that facilitate correct protein folding. However, all conditions could not yield soluble proteins.

Objective 2 **To develop a probe specific to mutans streptococci for rapid detection assay using CWBD of AmI conjugated with PAA-grafted MNPs.**

- **Preparation of CWBD of AmI**

Escherichia coli BL-21 carrying a recombinant CWBD plasmid were grown in LB broth at 37 $^{\circ}$C until reach OD_{600nm} of 0.4-0.6. The expression of 6xHis-tagged CWBD proteins was induced by the addition of 1 mM isopropyl-ß-D-thiogalactoside (IPTG) and incubated at 15 $^{\circ}$C for 24 h. The proteins then were purified using HIS-Select$^{®}$ Nickel Affinity Gel (Sigma-Aldrich Corp., St. Louis, MO, USA) under native condition. The active fractions were pooled and dialyzed against 0.1 M phosphate buffer (pH 6.8). The concentration of protein was measured by using the Bradford protein assay.

- **Preparation of PAA-grafted MNPs**

Magnetic nanoparticles (MNPs) were synthesized by solvothermal method (Shim et al., 2014). Briefly, 1.35 g of iron chloride hexahydrate (FeCl$_3$•6H$_2$O) was dissolved in 20 mL of ethylene glycol with vigorous stirring. After the solution became colorless, 3.6 g of sodium acetate (NaAc) was added with continuous stirring for 30 min. at room

temperature. Then the mixture was transferred into a 50 mL Teflon-lined stainless-steel autoclave and reacted at 200 °C for 12 h. After the reaction completed, the autoclave was cooled to room temperature. The formed black Fe_3O_4 nanoparticles were collected under external magnetic field, rinsed with ethanol for 10 times, and dried at 60 °C to yield black powder of MNPs (69% yield).

Poly(acrylic acid) (PAA) was grafted on MNPs surface by a grafting form method. Firstly, 0.1 g of MNPs was dispersed ultrasonically in 100 mL of 9:1 (v/v) ethanol-water mixture containing 1 mL of ammonia solution for 30 min. One mL of 3-aminopropyltriethoxysilane (APTES) was added dropwise to the dispersed solution. The mixture then was stirred at the room temperature for 12 h. The APTES-modified MNPs were isolated using an external magnet, washed with ethanol for several time and then were vacuum dried. The APTES-modified were immersed in 20 mL DMF containing ACVA (37.5 mmol), DCC (47.0 mmol) and DMAP (3.74 mmol) at room temperature for 20 h under nitrogen atmosphere. After a reaction was complete, the product was rinsed thoroughly with DMF and ethanol for four times each. The obtained initiator-immobilized MNPs were then placed in a vial containing ACVA (0.5 mmol), CTA (5.0 mmol) and acrylic(acid) (0.8 mol) in 10 mL of milli-Q water. The mixture then was heated at 70 °C for 20 h under nitrogen atmosphere. The resulting surface-grafted PAA were removed from the vial and washed with ethanol and milli-Q water, respectively.

- **Conjugation of CWBD on PAA-grafted MNPs by the EDC/NHS coupling method**

Five mg of PAA-grafted MNPs were dispersed in 1 mL of 0.01 M PBS (pH 7.4). 0.1 mL of EDC (2 M) and NHS (0.5 M) were added to the mixture and then incubated

for 30 min at room temperature with shaking (240 rpm). 0.1 mL of CWBD proteins (1 mg/mL) were added to the mixture and incubated for 24 h at 4 °C. Finally, CWBD-conjugated MNPs isolated using an external magnet and rinsed with 0.01 M PBS (pH 7.4) and milli-Q water, respectively. CWBD-conjugated MNPs were stored at 4 °C until use.

<u>Objective 3</u> To evaluate the specific affinity of CWBD-conjugated MNPs to mutans streptococci.

The binding affinity of CWBD-conjugated MNPs was tested with *S. mutans* UA159, *S. sobrinus* OMZ176, *S. sanguinis* ATCC10556 and *S. salivarius* DMST18781. Bacteria were cultured in brain hear infusion (BHI) broth at 37 ^{o}C, 5% CO_2 with shaking (180 rpm) for overnight. The optical density of overnight cultures was measure at 600 nm (OD_{600nm}) and then was adjusted to OD_{600nm} of 0.1. The adjusted cultures were then incubated at 37 ^{o}C, 5% CO_2 with shaking (180 rpm) to obtain OD_{600nm} of 0.5 ($\approx 10^{10}$ CFU/ml; log phase). The cultures then were diluted to 10^{6} CFU/ml with 0.01 M PBS (pH 7.4). The colony count was performed to confirm the number of bacteria (before treatment).

0.1 mL of each tested bacterial cultures was mixed with 0.2 mL of CWBD-conjugated MNPs solution (0.15 mg/mL in 0.01 M PBS (pH 7.4)) and added 0.01 M PBS (pH 7.4) upto 1 mL in a 1.5 mL tube. For a control group, bacterial cells were substituted with PBS. All tubes were then incubated for 30 min at 4°C. After incubation, a magnet was used to isolate CWBD-conjugated MNPs from a mixture. The supernatant liquids were transferred to a new tube and then plated on BHI agar plates to perform

the colony count (after treatment). CWBD-conjugated MNPs were re-suspended in 0.1 mL of 0.01 M PBS (pH 7.4). 50 µL of the suspensions were filtered through a 0.8 µm nitrocellulose membrane with a vacuum. The bacterial-bound CWBD-conjugated MNPs were then retained on the membrane since their size was bigger than the membrane pore size. The color intensity of the spots on a membrane, which reflected the amount of bacteria, was quantified by scion image for windows, beta 4.0.2. The experiment was performed 6 times.

Objective 4 **To examine the quantitative ability of the chromogenic assay in the detection of mutans streptococci**.

The experimental protocol is same as in objective 2 except the dilution process. To examine the quantitative ability, the log phase cultures were serially diluted ten-fold from 10^7 to 10^2 CFU/ml with 0.01 M PBS (pH 7.4). After filtration, the color intensity of the spots on a membrane was quantified by scion image for windows, beta 4.0.2. The experiment was done in triplicate. The capture efficiency (CE) of the CWBD-conjugated MNPs was determined by colony count (CFU). The CE was calculated using the formula:

$$CFU_{before}\text{-}CFU_{after}/CFU_{before} \text{ x } 100.$$

Furthermore, the relationship between the color intensity and the number of bacteria was analyzed by a simple linear regression.

Results and discussion

CWBD-conjugated MNPs showed a substrate-specific binding ability towards mutans streptococci but not to *S. sanguinis* and *S. salivarius*.

A substrate-specific binding ability of CWBD-conjugated MNPs was determined against 4 oral streptococci (10^6 CFU/ml) and a control without bacteria. The color intensity of CWBD-conjugated MNPs on a filter membrane with 0.8 µm pore size represents bacteria-bound CWBD-conjugated MNPs. *S. mutans* UA159 and *S. sobrinus* OMZ176 which are cariogenic streptococci exhibited higher signal intensity than the other two oral streptococci (Fig. 1). Based on the color intensity, we could apparently differentiate cariogenic streptococci from others with the naked eye.
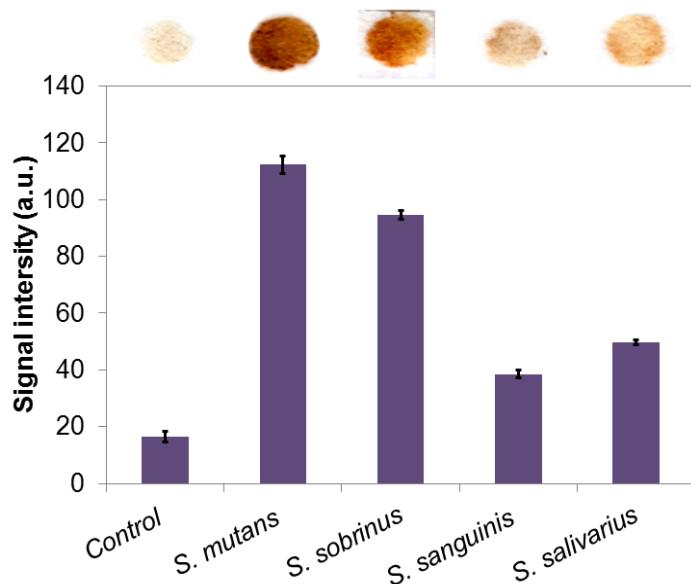
Fig.1 Signal intensity of bacteria-bound CWBD-conjugated MNPs on a nitrocellulose membrane.

Moreover, we determined the number of bacteria before and after binding to CWBD-conjugated MNPs by counting the colony. As seen in the figure 2, the number of *S. mutans* UA159 and *S. sobrinus* OMZ176 was decreased dramatically after binding to

the CWBD-conjugated MNPS but not found this finding in *S. sanguinis* ATCC10556 and *S. salivarius* DMST18781.
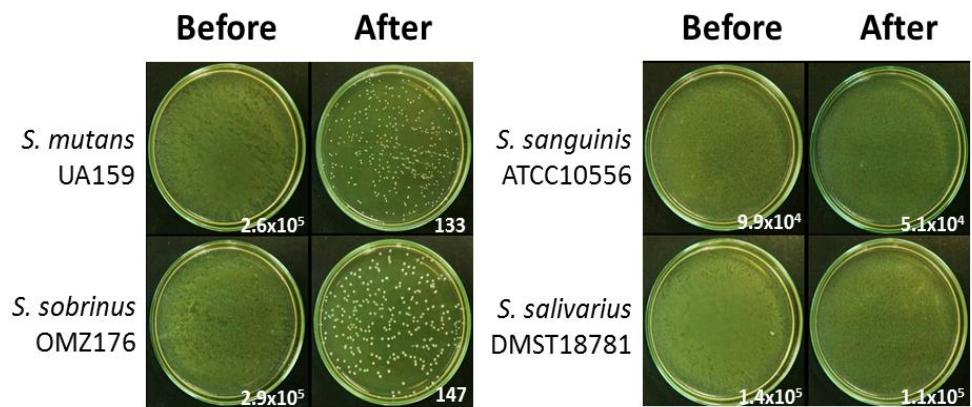
Fig. 2 Photographs of tested oral streptococci colonies on BHI agar plate before and after binding to CWBD-conjugated MNPs. The white numbers represent the number of bacteria (CFU/ml).

The capture efficiency of the CWBD-conjugated MNPs for *S. mutans* and *S. sobrinus* were 77 and 69%, respectively, whereas that for *S. sanguinis* and *S. salivarius* (non-MS) was 38 and 15%, respectively.

CWBD-conjugated MNPs exhibits semi-quantitative measurement ability in cariogenic streptococci detection.

Since the caries risk prediction relates to the number of mutans streptococci, the semi-quantitative measurement ability of CWBD-conjugated MNPs was determined. We found that the color intensity of bacteria-bound CWBD-conjugated MNPs remaining on 0.8 µm pore size membrane show a strong positive relationship with the number of bacteria (Fig. 3). Moreover, the regression coefficients of *S. mutans* and *S. sobrinus* indicated that the color intensity increased by 11.35 and 10.92, respectively for each log

CFU/ml which are approximately 3 times of those of *S. sanguinis* and *S. salivarius* (b = 3.70 and 3.81, respectively) (Fig. 3).
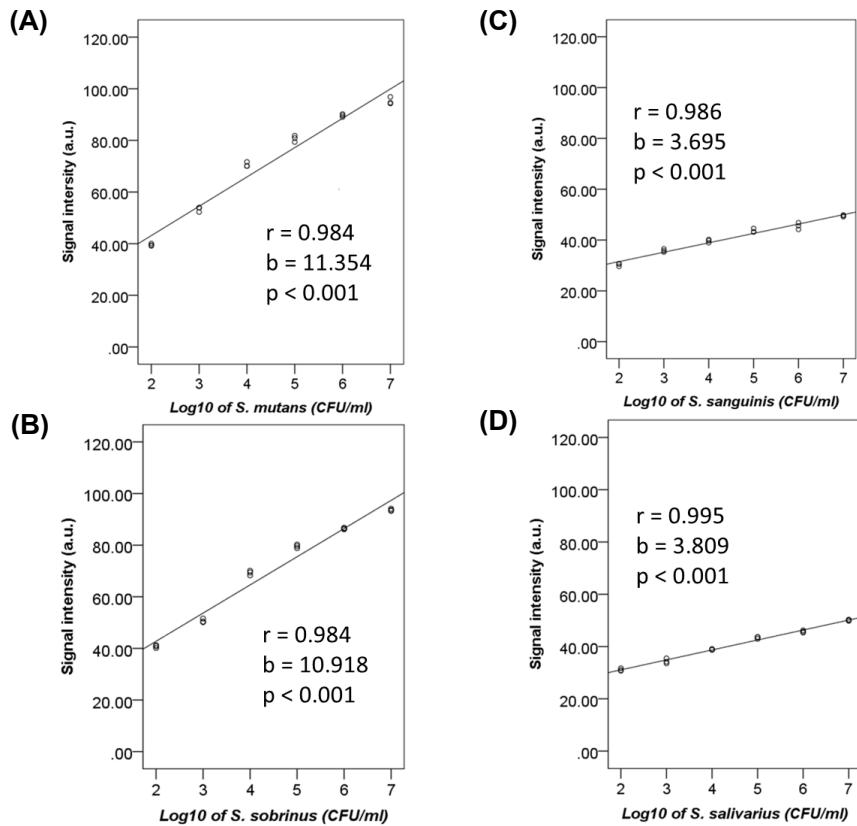
Fig. 3 Relationship between signal intensity and log 10 (CFU/ml) of *S. mutans* UA159 (A), *S. sobrinus* OMZ176 (B), *S. sanguinis* ATCC10556 (C) and *S. salivarius* DMST18781.

r = correlation coefficient and b = regression coefficient.

Conclusion

The colorimetric assay using CWBD-conjugated MNPs had an efficiency to bind to MS and distinguish MS from the other two predominant streptococcal species in oral cavity. Moreover, the color intensity of CWBD-conjugated MNPs can represent the semi-quantitative number of bacteria in range using in caries risk assessment (CRA).

Therefore, it could be developed into a visual MS detection tool for CRA. However, to develop this method for clinical applications, we need to perform the experiment using saliva, which is more complex than single species culture.

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Outputs (Supporting documents: Appendix)

1. International conference: Poster presentation (the 31st International Association of Dental Research South East Asian Division Joint Annual Meeting, August 10th-13th, 2017, at the Taipei International Convention Center,Taipei, Taiwan)

2. Patent:

    a. 1 patent pending (เลขที่คำขอ 1601003554)

    b. 1 petty patent (เลขที่อนุสิทธิบัตร 13042)

3. Manuscript submission:

    a. Biosensors and Bioelectronics – Rejected (the paper does not fully fall within the scope of our journal)

    b. Analytica Chimica Acta – Rejected (your manuscript is not suitable for publication in ACA)

    c. Talanta – Rejected (it does not appear to meet the aims and scope of TALANTA)

    d. Analyst – Rejected (I regret to inform you that I do not find your work suitable for publication in Analyst)

    e. Analytical Methods (Impact factor 2016 = 1.900) – Accepted

Appendix

CONTROL ID: 2757733

TITLE: Colorimetric Detection of Mutans Streptococci Using Magnetic Nanoparticles

Objectives: Chair-side detection of mutans streptococci (MS: Streptococcus mutans and Streptococcus sobrinus) is an important aid to clinical dental caries risk assessment. Automutanolysin (Aml) is a peptidoglycan hydrolase whose cell wall binding domain (CWBD) has substrate specificity towards MS. This study aimed to develop a visual detection assay using magnetic nanoparticles (MNPs) conjugated with CWBD of Aml.

Methods: MNPs were grafted with poly(acrylic-acid) providing active carboxyl-groups for conjugation with CWBD of Aml. To determine the binding specificity to MS, the CWBD-conjugated-MNPs were mixed with pure cultures of standard strains of 4 different species of streptococci. Bacteria-bound MNPs were then separated from unbound-cells by magnets and re-suspended in phosphate buffer saline. The suspension was then filtered through a membrane (pore-size 0.8μm). The color intensity of MNPs remained on the membrane represents the quantity of bound-bacteria. Moreover, to determine the capture efficiency (CE) of the CWBD-conjugated MNPs, bacterial cultures were incubated with the CWBD-conjugated MNPs and the amounts of cells before and that remained after the incubation were determined by colony count (CFU). The CE was calculated using the formula: $CFU_{before}-CFU_{after}/CFU_{before}$ x 100.

Results: The color intensity of CWBD-conjugated MNPs bound to MS was higher than those bound to the non-MS. This result implies that the CWBD-conjugated MNPs have greater binding specificity towards MS than non-MS. Moreover, the capture efficiency of the CWBD-conjugated MNPs for S. mutans and S. sobrinus were 77 and 69%, respectively, whereas that for S. sanguinis and S. salivarius (non-MS) was 38 and 15%, respectively. These values confirmed the specificity of the CWBD-conjugated MNPs towards S. mutans and S. sobrinus, but not to non-MS.

Conclusions: These results suggest that the colorimetric assay using MNPs conjugated with CWBD can specifically bind to MS and could be developed into a visual MS detection tool.

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Pre-Registration: Pre-Registration

Colorimetric Detection of Mutans Streptococci Using Magnetic Nanoparticles

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Introduction

Chair-side rapid mutans streptococci detection
• An important aid to clinical dental caries risk assessment.

Automutanolysin (Aml)
• A peptidoglycan hydrolase, an enzyme that cleaves glycosidic bonds within peptidoglycan structure of bacterial cell wall.
• Two domains: a C-terminal catalytic domain and an N-terminal cell wall-binding domain (CWBD).
• Its CWBD has substrate specificity towards cariogenic streptococci; *S. mutans* and *S. sobrinus*.

Objective

To develop a visual detection assay using magnetic nanoparticles (MNPs) conjugated with CWBD of Aml.

Materials and Methods

Immobilization of CWBD on MNPs-PAA

MNPs-PAA → (EDC, NHS / NH₂-CWBD) → **CWBD-conjugated MNPs**

EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS = N-hydroxysuccinimide
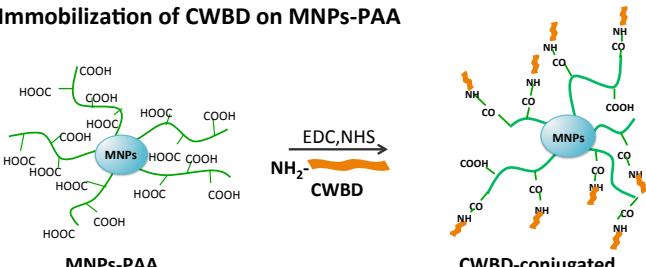
Determination of binding ability and specificity of CWBD-conjugated MNPs against MS and other oral streptococci
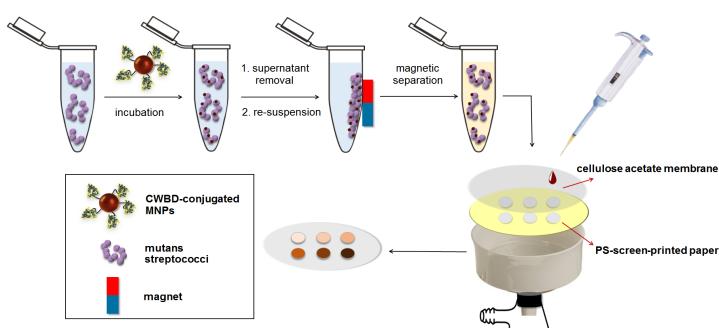
Capture efficiency (%) = {($C_{before} - C_{after}$)/C_{before}} x 100

Results

The color intensity of CWBD-conjugated MNPs bound to MS was higher than those bound to the non-MS.
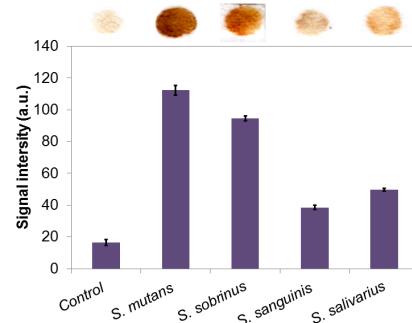
The number of MS after binding to the CWBD-conjugated MNPs dramatically decreased compared to those before binding whereas non-MS were not.
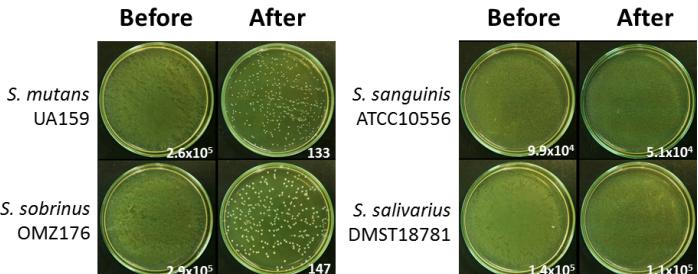
	Before	After
S. mutans UA159	2.6x10⁵	133
S. sobrinus OMZ176	2.9x10⁵	147
S. sanguinis ATCC10556	9.9x10⁴	5.1x10⁴
S. salivarius DMST18781	1.4x10⁵	1.1x10⁵

The capture efficiency of the CWBD-conjugated MNPs for *S. mutans* and *S. sobrinus* was 77% and 69%, respectively, whereas that for *S. sanguinis* and *S. salivarius* was 38% and 15%, respectively.

Conclusion

• These results suggest that the colorimetric assay using CWBD-conjugated MNPs can specifically bind to MS and could be developed into a visual MS detection tool.
• Future studies: detection of mutans streptococci in saliva samples

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Separation and Detection of Mutans Streptococci by Magnetic Nanoparticles Stabilized with Cell Wall Binding Domain-Conjugated Polymer

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The number of salivary mutans streptococci (MS: *Streptococcus mutans* and *Streptococcus sobrinus*) is used in dental caries risk assessment. In this study, a simple, yet effective assay was developed for MS detection. Magnetic nanoparticles (MNPs) were first grafted with poly(acrylic acid) that bears active carboxyl groups available for conjugation with the cell-wall-binding-domain (CWBD) of automutanolysin which specifically binds to MS. The binding efficiency of CWBD-conjugated MNPs to MS was tested with pure cultures of streptococcal standard strains. After mixing CWBD-conjugated MNPs with culture, bacteria-bound particles were separated from unbound cells using a magnet and filtered through a cellulose acetate membrane (pore-size 0.8 µm). The color intensity of particles remaining on the membrane represents the number of bound bacteria. The CWBD-conjugated MNPs showed higher efficiency in binding to *S.mutans* and *S.sobrinus* than to non-mutans streptococci (*S.sanguinis* and *S.salivarius*) with capture efficiency of 77 and 69% for MS, and 38 and 15% for non-MS, respectively. Moreover, this method can quantify the number of MS in the range of 10^2–10^7 colony-forming units (CFU)/mL, which covers the range of MS levels used in caries risk assessment. The calculated limit of detection of the assay was 16 and 72 CFU/mL for *S. mutans* and *S. sobrinus*, respectively. Furthermore, the CWBD-conjugated MNPs could be used to efficiently quantify the number of MS in human saliva samples containing highly complex mixtures of bacterial species. These results suggest that the assay could be applicable as a simple tool for MS determination in not only clinical settings but also community fields without clinical expert requirement.

Introduction

Dental caries is one of the most prevalent infectious diseases in mankind. Although it is not life-threatening, it can cause pain and lead to severe infection. [1] Moreover, the disease impacts on quality of life, especially in children. [2, 3] While the direction

of caries management has been gradually shifting from treatment by restoration to prevention in recent decades, the prevalence of dental caries remains high, especially in underprivileged populations worldwide .This situation is caused by the failure of treatments and ineffective prevention strategies. Caries risk assessment is a tool to help solve the problem since it gives information on individual risk of caries development and the dominant etiological factors in each patient. This information allows dentists to select an individualized treatment and prevention program for the patient. [2, 4]

The number of mutans streptococci (MS; *S mutans* and *S . Sobrinus*) in saliva is one of the factors determining the risk of caries development since there is much evidence showing association between the amount of MS and caries status.[5] The culture-based method is still widely used to determine the level of salivary MS, and is considered the gold standard.[6] However, the method requires laboratory skill, equipment, and also time for bacterial growth, so it is impractical in clinical settings and for community field studies. To overcome these limitations, a number of commercial chair-side kits have been developed.[7, 8] However, despite their ease of use and their equivalent efficiency to the conventional culture-based method, these kits still require an incubator and time to grow bacteria. Recently, a commercial *S .mutans* rapid-detection kit

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†These two authors contributed equally and are considered co-first authors.
†Electronic Supplementary Information (ESI) available: Characteristics of CWBD, XRD pattern of bare MNPs, FTIR spectra, average size, zeta potential and TGA data of MNPs both before and after stepwise chemical modification, Explanation on how to determine limit of detection and capture efficiency, Photographs of oral streptococci colonies before and after binding to CWBD-conjugated MNPs. See DOI: 10.1039/x0xx00000x

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based on antigen-antibody reaction has become available. Gao and colleagues reported that this kit could determine the number of *S. mutans* in saliva samples more accurately than the culture-based method. [9] Nevertheless, classification of caries risk is still based on the salivary MS levels obtained from the culture-based method. A level of MS >10^5 colony forming units (CFU)/mL is considered as high-risk for caries, whereas that below 10^4 CFU/mL is considered low-risk.[10, 11]

Here, to develop a specific detection system for MS, we exploit the binding specificity of the enzyme automutanolysin, a peptidoglycan hydrolase produced by *S. mutans*. The enzyme selectively digests MS.[12] The substrate specificity of automutanolysin towards MS is conferred by the cell wall binding domain (CWBD) of the enzyme (unpublished observation). Thus, the CWBD could be a good probe for MS detection.

Magnetic separation in combination with size-selective membrane filtration was recently introduced as a rapid, versatile, simple, low-cost and effective method for bacterial separation/detection.[13, 14] Magnetic nanoparticles (MNPs) usually appear in a magnetite form (Fe_3O_4) which responds to a magnetic field.[15] Moreover, their small sizes (5 to 500 nm diameter) result in a large surface to volume ratio available for conjugation of probes (mostly antibodies) specific for bacteria, which enhances the bacterial capture efficiency.[16-19] Based on these properties of MNPs and the binding specificity of the probe, target bacteria can be isolated from contaminants using an external magnet.[13, 20, 21] After magnetic separation, the targeted bacterial suspension is filtered through a membrane with a specific pore size. If the MNPs conjugated with the probe capture target bacteria, their size increases, so they are retained on the membrane.[14, 22] Since MNPs are brown, the bacteria-bound MNPs remaining on the membrane can easily be detected by the naked eye.

A number of polymers such as dextran,[23] polystyrene (PS),[24, 25] and poly(acrylic acid) (PAA)[26, 27] have been coated onto MNPs to increase their stability and dispersibility. The polymeric stabilizer can increase the affinity of MNPs for a specific solvent and inhibit particle aggregation.[28-31] Polymer-grafted MNP nanocomposites form very stable dispersions in organic solutions.[32-34] Among the most effective methods for preparing polymer-coated MNPs is surface functionalization by grafting of polymer, particularly via the "grafting from" approach or surface-initiated polymerization. This strategy involves the formation of a covalently bound polymeric layer growing from a surface-attached initiator. This method provides high grafting density with controlled polymer molecular weight so as the thickness of coated polymer layer if controlled polymerization process (i.e. atom transfer radical polymerization (ATRP), reversible addition fragmentation chain transfer (RAFT) polymerization) is employed.[32, 35-37]

Based on these previous studies, this research aimed to develop CWBD-conjugated MNPs as a simple tool for MS detection based on magnetic separation and size-selective membrane filtration. MNPs were grafted with PAA via surface-initiated RAFT polymerization to yield PAA-grafted MNPs. CWBD was then conjugated to the carboxyl groups of PAA via

1-ethyl-3-3-dimethylaminopropyl(carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) coupling chemistry. The CWBD-conjugated MNPs specifically bound to the target bacteria and then an external magnetic field was applied to separate and concentrate the MNPs-bound captured bacteria from contaminants. The bacteria-bound CWBD-conjugated MNPs were subsequently filtered through a cellulose acetate membrane. With appropriate pore size of the membrane, the bacteria-bound CWBD-conjugated MNPs remained on the membrane and could be detected by the naked eye.

Experimental

Materials

Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) was purchased from Merck (Germany). MNPs were prepared via a solvothermal method following a published procedure.[38] 3-Aminopropyltriethoxysilane (APTES), 4,4-azobis(4-cyanovaleric acid) (ACVA), 4-cyano-4-(phenylcarbonothio) pentanoic acid, 4-(dimethylamino)pyridine (DMAP), N,N'-dicyclohexylcarbodiimide (DCC), dimethylformamide (DMF), EDC, NHS, acrylic acid (AA), sodium acetate, and ethylene glycol were purchased from Sigma-Aldrich (USA). Phosphate buffered saline (PBS, pH 7.4), and cellulose acetate membrane (CA membrane with a 0.2, 0.45, 0.8 and 1.2-μm pore sizes were purchased from Merck Millipore (USA). All culture media except Mitis Salivarius Bacitracin (MSB) agar were purchased from HiMedia (India). Mitis salivarius agar and bacitracin were bought from Becton, Dickinson and Company (USA) and Sigma-Aldrich respectively. Materials for protein expression and purification, isopropyl-β-D-thiogalactoside (IPTG), HIS-Select® Nickel Affinity Gel, imidazole, dialysis bags (MWCO 10 kDa) and Bradford Protein Assay reagent were bought from Calbiochem (India), Sigma-Aldrich, Affymetrix (USA), Thermo Scientific (USA) and Bio-Rad (USA), respectively. All reagents and materials were analytical grade and used without further purification. Ultrapure distilled water was obtained using a Millipore Milli-Q system (USA).

Characterization of MNPs

The crystal structure of MNPs was characterized by X-ray diffraction (XRD). The XRD pattern was obtained using a Rigaku RINT-2000 diffractometer (Japan) using CuKα radiation (λ = 1.5418 Å). Infrared spectra of surface-modified MNPs prepared as KBr disks were obtained from a Fourier Transform-Infrared (FTIR) spectrometer (Nicolet, USA), model Impact 410, with 32 scans at resolution 4 cm^{-1}, in the frequency range 400-4000 cm^{-1}, using a TGS detector. The morphology of MNPs before and after modification was examined by transmission electron microscopy (TEM) using a Philips Tecnai 12, FM 208 instrument (USA) at 100 kV acceleration. Negative staining was performed by dropping 1% (w/v) aqueous solution of phosphotungstic acid on the MNPs deposited on the carbon-coated copper grid. The excess solution was blot-dried by filter paper. The sample was then air-dried in the dark overnight before subjected to TEM analysis. Thermogravimetric analysis (TGA) was

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performed on a SDTA 851 (Mettler-Toledo, USA) in the range of 32–900 °C at a heating rate of 20 °C/min under nitrogen. The size and zeta potential of the MNPs were measured by dynamic light scattering using a Malvern Nano ZS90 instrument (UK) at room temperature at a scattering angle of 90°.

Preparation of CWBD of automutanolysin

Escherichia coli BL-21 carrying a recombinant CWBD-expressing plasmid were grown in Luria-Bertani broth at 37 °C until an $OD_{600\ nm}$ of 0.4–0.6 was reached. The expression of 6×His-tagged CWBD protein was induced by addition of 1 mM IPTG and incubation of cells at 15 °C for 24 h. The proteins were then purified using HIS-Select® Nickel Affinity Gel in native conditions. The active fractions were pooled and dialyzed against 0.1 M phosphate buffer, pH 6.8. The protein concentration was measured by the Bradford protein assay. Sequence of CWBD together with schematic plasmid map including CWBD and the expressed CWBD protein on SDS-PAGE (Fig.S1) are shown in ESI†.

Preparation of PAA-grafted MNPs

PAA was grafted onto the surface of MNPs via a "grafting from" method. MNPs were first immobilized with APTES. Briefly, 0.1 g of MNPs was dispersed ultrasonically in 100 mL of 9:1 (v/v) ethanol-water mixture with 1 mL of added ammonia solution (38%) for 30 min. To the above dispersed solution, 1 mL of APTES was added dropwise, and the resulting mixture was stirred at room temperature for 12 h. Thereafter, the APTES-modified MNPs obtained were magnetically concentrated, washed thoroughly with ethanol, and vacuum dried. The APTES-modified MNPs were immersed in 20 mL DMF containing ACVA (37.5 mmol), DCC (47.0 mmol) and DMAP (3.74 mmol) at room temperature for 20 h under a nitrogen atmosphere. After the reaction was complete, the product was rinsed thoroughly with DMF and ethanol four times each. The obtained initiator-immobilized MNPs were then placed in a vial containing ACVA (0.5 mmol), CTA (5 mmol), and AA (0.8 mol) in 10 mL of Milli-Q water. The vial was then placed in a preheated oil bath at 70 °C for 20 h under a nitrogen atmosphere. The resulting PAA-grafted MNPs were removed from the vial and washed with ethanol and Milli-Q water, respectively. The synthetic scheme for the preparation of PAA-grafted MNPs via surface-initiated RAFT polymerization
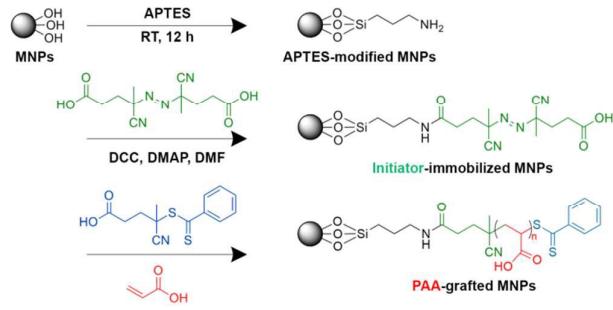
Scheme 1 Preparation of PAA-grafted MNPs via surface-initiated RAFT polymerization.

is shown in **Scheme 1**.

Immobilization of CWBD on PAA-grafted MNPs

PAA-grafted MNPs were conjugated with the CWBD of automutanolysin via the EDC/NHS coupling method, as follows. PAA-grafted MNPs (5 mg) were dispersed in 1 mL 0.01 M PBS, pH 7.4. To this, 0.1 mL of EDC (2 M) and 0.1 mL of NHS (0.5 M) were added, and then the mixture was incubated for 30 min at room temperature at 240 rpm on a rotary shaker. CWBD (0.1 mL, 1 mg/mL) was added to the mixture and it was incubated for 24 h at 4°C. Finally, the CWBD-conjugated MNPs were isolated using a magnet and rinsed with 0.01 M PBS, pH 7.4 and Milli-Q water, respectively. The product was stored at 4 °C until use.

Bacterial preparation

S. mutans UA159, *S. sobrinus* OMZ176, *S. sanguinis* ATCC10556 and *S. salivarius* DMST18781 were cultured in brain heart infusion broth at 37 °C in a 5 %CO_2 atmosphere with shaking at 180 rpm for 24 h. The optical density of 24-h culture was measured at 600 nm (OD_{600nm}). The cultures were then diluted to OD_{600nm} = 0.1 and incubated for 2.5 h to obtain an OD_{600nm} of 0.5 (~10^{10} CFU/mL). After that, the bacterial cultures were 10-fold serially diluted from 10^7 to 10^2 CFU/mL with 0.01 M PBS, pH 7.4. Colony counting was performed to verify the number of bacteria.

Determination of bacteria captured by CWBD-conjugated MNPs

The efficiency of CWBD-conjugated MNPs in capturing *S. mutans* UA159, *S. sobrinus* OMZ176, *S. sanguinis* ATCC10556, and *S. salivarius* DMST18781 was determined .Briefly, 0.1 mL bacterial suspensions in the range 10^2 to 10^7 CFU/mL were transferred to a 1.5-mL tube containing 0.2 mL of CWBD-conjugated MNP suspension (0.15 mg/mL in 0.01 M PBS, pH 7.4) and then incubated for 30 min at 4 °C .After incubation, the tube was placed on a magnet for 10 min to isolate and concentrate the bacteria-bound CWBD-conjugated MNPs. The supernatant was removed and bacteria-bound CWBD-conjugated MNPs were resuspended in 0.1 mL of 0.01 M PBS, pH 7.4. A piece of filter paper (Whatman #1, i.d = .125 mm) was screen-printed with PS in a pattern that leaves circular areas of 5 mm diameter uncovered with PS, using a previously published method.[39] For selective filtration, a CA membrane with selected pore size (0.2, 0.45, 0.8, or 1.2 µm) was placed on top of the PS-screen-printed paper in a Büchner funnel equipped with an Erlenmeyer flask and an aspirator. The membrane was wetted with 0.01 M PBS, pH 7.4. Fifty microliters of the bacteria-bound CWBD-conjugated MNP suspension was then pipetted onto the membrane in a position that exactly matched the circular area of the PS-screen-printed paper underneath under vacuum. The overall concept for bacterial detection is illustrated in **Scheme 2**.

The color intensity on the membrane, which reflected the amount of bacteria in the sample, was quantified and averaged

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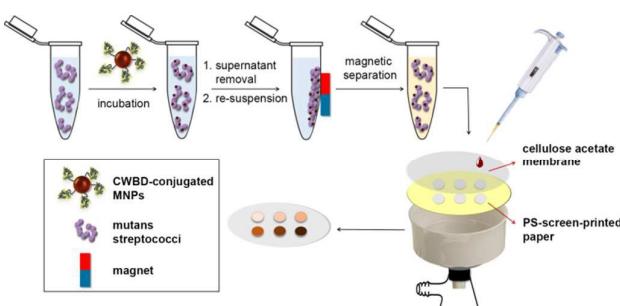
Scheme 2 Schematic representation of the stepwise procedure for colorimetric detection of mutans streptococci by CWBD-conjugated MNPs based on magnetic separation and selective filtration.

from three independent samples using scanned images recorded on an Epson Perfection V33 scanner in 24 bit Professional Mode. The brightness/contrast/resolution was set to 128/128/300. The images were saved as TIFF files. The intensity of each spot was determined using Scion image software by first converting to grayscale at 300 dpi. Intensity measurements were carried out using the Line tool to select an area for analysis to obtain profile images. The amount of bacteria was determined from a standard curve generated from the intensity as a function of known bacterial concentration.

Study population and saliva collection

The study protocol was approved by the Ethics Committee at the Faculty of Dentistry, Chulalongkorn University (HREC-DCU-P 2016-013). Sixteen healthy adult volunteers were recruited from among dental students at the Faculty of Dentistry, Chulalongkorn University and workers at a private company. Saliva samples were collected following the procedures of Wennerholm and Emilson with some modifications.[40] Briefly, all volunteers were informed not to brush their teeth and to refrain from having breakfast before collection of the saliva. Stimulated saliva was collected while chewing a piece of paraffin film for 10 min. The collected saliva was immediately placed on ice, delivered to the laboratory directly, kept at 4 °C, and used the same day.

Determination of the number of salivary mutans streptococci

Conventional culture-based method. Saliva was tenfold serially diluted with sterile 0.01 M PBS, pH 7.4, and then 100 μL of each dilution was spread onto a MSB agar plate. The MSB plates were incubated at 37 °C in 5% CO_2 for 24–48 h. After incubation, the number of mutans streptococcus was determined under a stereomicroscope.

CWBD-conjugated MNP assay. To determine the number of bacteria in saliva using CWBD-conjugated MNPs, 800 μL of stimulated saliva was treated by adding 200 μL of 1 M citric acid and 50 μL of 0.1 M sodium hydroxide[9] and then boiled for 20 min to denature proteins in the saliva. After that, 100 μL of treated saliva was mixed with 200 μL CWBD-conjugated MNP suspension (0.15 mg/mL in 0.01 M PBS, pH 7.4) and sterile 0.01 M PBS, pH 7.4, was added to make 1 mL total volume in a 1.5-

mL test tube. The mixture was then incubated at 4°C for 30 min. Magnetic separation and selective filtration of the bacteria-bound CWBD-conjugated MNPs were performed by the same procedure as described earlier.

Results and discussion

Preparation and characterization of PAA-grafted MNPs and CWBD-conjugated MNPs

MNPs were synthesized by a solvothermal method. As displayed in **Fig. S2** (ESI†), an XRD pattern of the MNPs showed diffraction peaks at $2\theta = 30.12$, 35.48, 43.12, 57.02 and 62.62°, which can be assigned to (311), (440), (220), (511), and (400) planes of Fe_3O_4, respectively. This characteristic coincides with that of standard magnetite (JCPDS card no. 19-0629). No other diffraction peaks corresponding to other iron oxides, such as α-Fe_2O_3 and γ-Fe_2O_3, could be detected, suggesting that the MNPs obtained are in the form of pure Fe_3O_4.

PAA-grafted MNPs were prepared by surface-initiated RAFT polymerization. The success of stepwise surface modification of MNPs was confirmed by FTIR as shown in **Fig. S3** (ESI†). A characteristic Fe–O vibration of the unmodified MNPs **(Fig. S3a)** was observed at 584 cm^{-1}. The spectrum of PAA-grafted MNPs **(Fig. S3b)** shows a band at 1740 cm^{-1}, which is assignable to C=O stretching of carboxyl groups of PAA. This signal disappeared upon CWBD conjugation **(Fig. S3c)**, indicating that an amide linkage was formed between carboxyl group of PAA and amino group of the enzyme.

Fig. 1 illustrates the morphology of the MNPs before and after modification as examined by TEM. The unmodified MNPs **(Fig. 1a)** were spherical with an average diameter of 328.2 ± 22.7 nm. The average diameter of the MNPs increased upon surface modification, to 357.4 ± 19.2 and 745.5 ± 29.9 nm for PAA-grafted MNPs **(Fig. 1b)** and CWBD-conjugated MNPs **(Fig.**
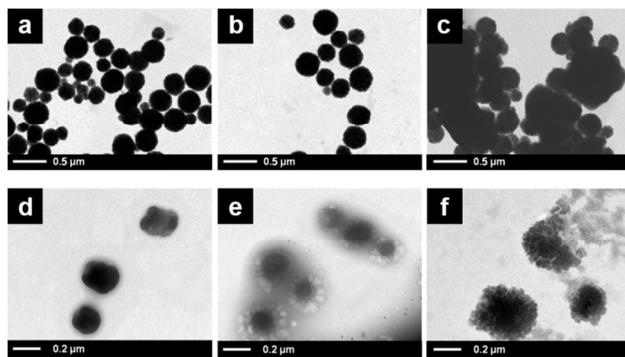

Fig. 1 Transmission electron micrographs of bare MNPs (a,d), PAA-grafted MNPs (b,e), and CWBD-conjugated MNPs (c,f) before (a-c) and after (d-f) negative staining (scale bars 0.5 μm (top) and 0.2 μm (bottom)).

1c), respectively. A higher degree of aggregation was also realized in the case of CWBD-conjugated MNPs. This may be ascribable to the conjugation with relatively large molecule of CWBD (MW =80 kDa). Negative-stained TEM images of both PAA-grafted MNPs and CWBD-conjugated MNPs demonstrating the organic shell layer of grafted PAA and conjugated CWBD, respectively are shown in **Fig.1e-f**.

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The particle size and zeta potential of the MNPs were also evaluated by DLS (**Table S1** (ESI†). In good agreement with the TEM data, the average size of the MNPs increased after PAA grafting and again after CWBD conjugation. The pK_a of PAA is about 4.5 [41] and the isoelectric point of CWBD determined by calculation is approximately 5.17, which should result in both PAA and CWBD being negatively charged in PBS solution at pH 7.4. For this reason, the PAA-grafted MNPs exhibited a negative zeta potential (-31.43 mV), verifying the presence of ionizable carboxyl groups of PAA surrounding the MNPs .After CWBD conjugation, the zeta potential of the MNPs became less negative (-14.40 mV), which was due to CWBD being less negatively charged than PAA. This set of data also indicated the successful immobilization of CWBD on the PAA-grafted MNPs.

The presence of organic components of PAA and CWBD on the PAA-grafted MNPs and CWBD-conjugated MNPs, respectively, was further confirmed by TGA analysis. As shown in **Fig. S4** (ESI†), the first weight loss of all MNPs took place below 200°C, which should originate from evaporation of water. CWBD-conjugated MNPs showed the highest weight loss (about 3.44%), implying that they contained the greatest bound water content. The second weight loss, occurring in the temperature range 300–600 °C, could be ascribed to the decomposition of organic content bonded to MNPs. The greater weight loss of 9.1 and 9.8% from PAA-grafted MNPs and CWBD-conjugated MNPs respectively, compared with 5.5% from MNPs, confirmed the presence of PAA and CWBD on the modified MNP forms. The third weight loss, appearing at the highest temperatures (>730 °C) in both PAA-grafted MNPs and CWBD-conjugated MNPs, is assigned as char of macromolecular organic content (PAA and CWBD) on the MNPs, because such weight loss was absent from the unmodified MNPs curve. As determined by Bicinchoninic acid protein assay, the quantity of immobilized CWBD was found to be 6.66 mg/g of PAA-grafted MNPs. The calculated immobilization efficiency was 32.8%.

Selection of filter membrane suitable for selective filtration assay

To determine an appropriate pore size of the filter membrane that would selectively retain bacteria-bound CWBD-conjugated MNPs, CA membranes with pore sizes in the range 0.2–1.2 μm were tested. The CWBD-conjugated MNPs would become larger in size once bound with bacteria and therefore retain on the membrane upon filtration if their size is larger than the pore diameter of the membrane. Since MNPs are brown, the bacteria-bound MNPs would appear as brown spot on the membrane. The higher bacterial concentration leads to higher level of MNPs aggregation and thus yielded the brown spot with greater intensity. As **Fig . 2** shows, even the unmodified MNPs (the smallest particles) could not go through the 0.2-μm pore-size filter membrane. This outcome corresponded to the results of TEM and DLS analyses, which indicated that the approximate diameter of the unmodified MNPs was 0.32 μm. When membranes with pore-size 0.45 μm were used, they could not separate bacteria-bound CWBD-conjugated MNPs

Pore size	MNPs	PAA-grafted MNPs	CWBD-conjugated MNPs	S.Salivarius CWBD-conjugated MNPs	S.Mutans CWBD-conjugae MNPs
0.2μm					
Intensity (a.u.)	132.88	128.73	131.70	119.83	129.92
0.45μm					
Intensity (a.u.)	21.70	81.12	118.60	118.16	120.18
0.8μm					
Intensity (a.u.)	21.70	30.15	40.13	80.54	141.65
1.2μm					
Intensity (a.u.)	10.23	12.74	16.14	23.18	31.84

Fig. 2 Color signal and corresponding intensity (arbitrary unit) of the brown spot on the CA membrane with various pore sizes after filtering MNPs, PAA-grafted MNPs and CWBD-conjugated MNPs both before and after bacterial capture.

from the unbound, CWBD-conjugated MNPs. This finding was consistent with TEM and DLS analyses showing the diameter of CWBD-conjugated MNPs was approximately 0.75 ± 0.03 and 0.63 ± 0.02 μm, respectively. The 0.8-μm pore-size filter membrane worked effectively; a difference in brown color intensity was apparent between bacteria-bound and unbound CWBD-conjugated MNPs. Moreover, higher color intensity of retained CWBD-conjugated MNPs when bound to S .Mutan (MS bacteria) implied greater binding efficiency of the CWBD toward S. mutans compared with S. salivarius (non-MS bacteria). All tested particles could pass through the 1.2-μm pore-size filter membrane. Based on these findings, the 0.8-μm pore-size filter membrane was selected for further investigation.

Determination of bacterial binding efficiency of CWBD-conjugated MNPs

Bacterial binding efficiency of the CWBD-conjugated MNPs was determined with S. mutans and S. sobrinus (MS bacteria) in the range 10^2–10^7 CFU/mL. **Fig. 3** shows the intensity of the brown spots produced by bacteria-bound CWBD-conjugated MNPs on 0.8-μm pore-size filter membranes. The intensity was converted to numeric values to generate a standard curve. The intensity of the colored spots increased linearly as a function of log (CFU/mL) for both bacteria **(Fig. 3a and 3b)**. The limit of detection (LOD) is the lowest detectable bacterial concentration, which is calculated following **Eq. S3** (ESI†). The background intensity (0 CFU/mL) was 23.82 ± 5.13, which resulted in LOD values of 16 and 72 CFU/mL for S. mutans and S. sobrinus, respectively.

Determination of bacteria capturing efficiency of CWBD-
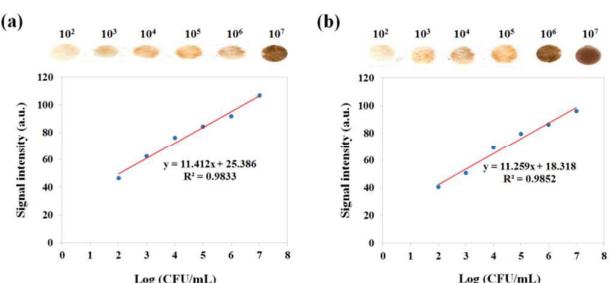
Fig. 3 Linear relationship between log (CFU/mL) and signal intensity (a.u.) for (a) S.mutans and (b) S.sobrinus.

conjugated MNPs for various streptococcal species

Capture efficiency of MS bacteria (*S. mutans* and *S. Sobrinus*) and non-MS bacteria (*S. sanguinis* and *S. salivarius*) by the CWBD-conjugated MNPs was determined. First, a colony counting assay was performed before (C_{before}) and after (C_{after}) binding of bacteria to the CWBD-conjugated MNPs. As shown in **Fig. S5** (ESI†), the number of *S. mutans* and *S. sobrinus* before binding to the CWBD-conjugated MNPs was approximately 10^5 CFU/mL, whereas after binding the numbers decreased to 133 and 147 CFU/mL, respectively. In contrast, the numbers of *S. sanguinis* and *S. salivarius* after binding to the CWBD-conjugated MNPs were only slightly decreased from the values before binding. The capture efficiency (CE) was calculated following **eq .S4** (ESI†) .The capture efficiency of the CWBD-conjugated MNPs for *S. mutans* and *S. sobrinus* was 77 and 69%, respectively, whereas that for *S. sanguinis* and *S. salivarius* was 38 and 15%, respectively. These values indicated the specificity of the CWBD-conjugated MNPs for MS bacteria (*S. mutans* and *S. sobrinus*), rather than non-MS bacteria (*S. sanguinis* and *S. salivarius*). This specificity towards MS bacteria was further confirmed by the selective filtration assay. As shown in **Fig. 4**, the signal intensities obtained from the interaction with non-MS species (*S. sanguinis* and *S. salivarius*) were much lower than those detected with the MS species (*S. mutans* and *S. sobrinus).*

Comparative investigation of bacterial detection by the magnetic separation/selective filtration method using CWBD-conjugated MNPs and the culture method in saliva samples

To develop the CWBD-conjugated MNPs for clinical application, we demonstrated that CWBD-conjugated MNPs can detect MS in human saliva, which contains a complex mixture of many bacterial species. Saliva samples were collected and then incubated with CWBD-conjugated MNPs. The number of MS was determined by comparing the detection intensity to the standard curve for *S. mutans.* Culture-based determination method was performed in parallel by inoculating the same saliva samples on MSB agar to determine the number of MS. As **Table 1** shows, the number of MS determined by the CWBD-conjugated MNPs method was similar to that obtained by the culture-based method. This similarity is important and useful, since the level of caries risk is classified based on the saliva MS
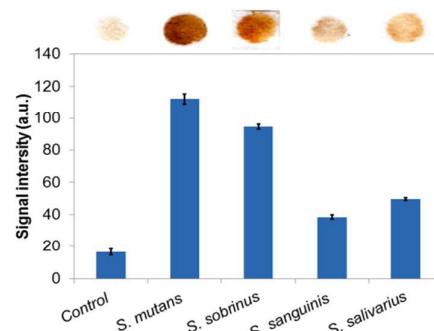

Fig. 4 Determination of binding specificity of the CWBD-conjugated MNPs to MS (*S .mutans* and *S .sobrinus*) and non-MS (*S .sanguinis* and *S .salivarius*) species.

Table 1 Number of mutans streptococci determined by the magnetic separation/selective filtration method using CWBD-conjugated MNPs in comparison with the culture-based method.

Saliva sample	Number of colonies (CFU/mL)	
	Magnetic separation/selective filtration method	Culture-based method
1	7.2×10^4	3.7×10^4
2	1.3×10^4	1.6×10^4
3	4.8×10^2	5.2×10^3
4	N/A	$< 10^2$
5	5.7×10^2	$< 10^2$
6	N/A	$< 10^2$
7	N/A	$< 10^2$
8	N/A	$< 10^2$
9	1.5×10^4	3.3×10^3
10	3.6×10^2	2.9×10^2
11	7.4×10^3	1.3×10^3
12	N/A	$< 10^2$
13	N/A	$< 10^2$
14	N/A	$< 10^2$
15	N/A	$< 10^2$
16	N/A	$< 10^2$

N/A = out of range of the standard curve (10^2–10^7 CFU/mL).

level obtained from the culture-based method. Together, our results indicated that the CWBD-conjugated MNPs have potential to be a simple colorimetric method to quantify the number of salivary mutans streptococci.

Conclusions

The present study demonstrated that PAA-grafted MNPs could be prepared by surface-initiated RAFT polymerization. They were successfully conjugated to the CWBD of automutanolysin by EDC/NHS coupling chemistry. The properties of CWBD-conjugated MNPs were characterized by XRD, FTIR, TEM, TGA and DLS. By combining bacterial binding to CWBD-conjugated MNPs with magnetic separation and selective filtration, we developed a colorimetric assay for quantitative MS detection in both pure cultures and human saliva samples. Most importantly, the assay could differentiate levels of salivary MS relevant for dental caries risk assessment. Without requiring for bacteria culturing or clinical experts, this effective assay is a promising tool for MS detection that can be easily implemented in not only clinical settings but also community fields. The simplicity of the assay would make dental caries risk assessment more accessible and efficient. The success of this work also suggests that the magnetic-based immunoassay may be applicable for detection of other bacteria in saliva, another biologically relevant matrix that has not been much explored.

Conflicts of interest

The authors declare no competing financial interest.

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Highlight statement to be included with GA (no more than 20 words)

Colorimetric assay based on magnetic separation/selective filtration can differentiate salivary mutans streptococci levels relevant for dental caries risk assessment.

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