DEVELOPMENT OF A SILVER NANOCLUSTER PROBE AGAINST FOXP3 FOR LIVE TREGS SORTING : USING MCF-7 AS A MODEL

by

LEE SHIN YONG

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LIST OF SYMBOL, ABBREVIATION AND ACRONYM

%	Percentage
Ag^+	Silver ion
AgNC	Silver nanocluster
$AgNO_3$	Silver nitrate
APC	Antigen-presenting cell
APS	Ammonium persulfate
Au^+	Gold ion
cAMP	Cyclic adenosine monophosphate
CD25	IL-2 receptor α chain
Cdna	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
dH_2O	Distilled water
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FACS	Fluorescence assisted cell sorting
FBS	Fetal Bovine Serum
Foxp3	Forkhead box protein 3
g	gram
GARP	Glycoprotein A repetitions predominant
GITR	Glucocorticoid-induced tumour necrosis factor receptor family gene
GITRL	Glucocorticoid-induced tumour necrosis factor receptor family gene ligand
HBS	HEPES buffered saline
hrs	hours
iTreg	Induced regulatory T cells
IL-10	Interleukin 10
LAP	Latency-associated peptide
mins	Minutes
mL	mililiter
mM	milimolar
Mg^{2+}	Magnesium ion
MHC	Major histocompatibility complex
mRNA	Messenger Ribonucleic Acid
$NaBH_4$	Sodium borohydride
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
Na_2HPO_4	Disodium hydrogen phosphate
NFAT	Nuclear factor of activated T-cells
Nrp-1	Neuropilin-1

nTreg	Natural regulatory T cells
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pTreg	Peripheral regulatory T cells
qRT-PCR	quantitative real-time polymerase chain reactioin
RNA	Ribonucleic acid
SD	Standard deviation
ssDNA	Single-stranded deoxyribonucleic acid
TBE	Tris-boric-EDTA
Tc	Cytotoxic T cells
Teff	Effector T cells
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TNF	Tumour necrosis factor
TNFRSF	Tumour necrosis factor receptor superfamily
Th	T helper
Th3	T helper 3 regulatory T cells
Th17	T helper 17
Tr1	Type 1 regulatory T cells
Tregs	Regulatory T cells
tTreg	Thymic Regulatory T cells
UV	Ultraviolet

PEMBENTUKAN PROBA NANOKLUSTER PERAK UNTUK FOXP3 BAGI PENGASINGAN SEL T REGULATOR HIDUP: MENGGUNAKAN SEL MCF-7 SEBAGAI SATU MODEL

ABSTRAK

DNA ber-templat Nanokluster Perak (AgNC) adalah kelas baru prob pendarfluor yang diberi penekanan beberapa tahun kebelakangan ini. Dengan ciri-ciri khas seperti bersaiz kecil, mempunyai kestabilan foto dan keterlarutan dalam air, prob pendafluor ini turut digunakan secara meluas dalam aplikasi diagnostik. Dengan menggunakan kelebihan AgNCs ini, kami mengadaptasikan pendekatan ini agar boleh digunakan untuk pengesanan sel T regulator (Tregs) melalui faktor transkripsi intraselular iaitu forkhead box P3 (Foxp3). Tregs adalah perantara penting homeostasis keimunan yang mengenakan supresi kepada sel efektor dalam penyakit autoimun dan menggalakkan pertumbuhan kanser. Pemencilan Tregs hidup adalah diperlukan untuk pencirian Tregs yang lebih baik. Buat masa ini, pengesanan Tregs bergantung pada pelbagai biomarker di permukaan sel dan satu biomarker intrasel, Foxp3. Pengasingan populasi tulen Tregs adalah mustahil buat ketika ini kerana pengesanan intrasel Foxp3 bergantung pada penetapan dan ketelusan sel yang menyebabkan kematian sel. Di sini kami memperkenalkan kaedah optimum untuk pengesanan mRNA sasaran Foxp3 dengan penempelan prob AgNC dan G-kaya kepada sasarannya. Sampel penempelan diperiksa di bawah cahaya UV dan keamatan pendafluor ditentukan oleh pendafluor spektroskopi. Kejayaan penghibridan tiga hala persimpangan dengan AgNC, G-kaya dan sasaran DNA / RNA Foxp3 menjana perubahan dalam pendafluor spektrum dengan peningkatan dalam keamatan pendafluor. AgNC dan G-kaya dipindahkan ke dalam sel MCF-7 (model sel yang mempunyai Foxp3) menghasilkan imej berpendafluor merah yang boleh diverifikasikan dengan menggunakan kaedah sitometri aliran. Ringkasannya, pendekatan nanokluster perak berpotensi untuk mengesan asid

nukleik Foxp3 intrasel sebagai suatu kaedah pengesanan intraselular yang tidak membunuh untuk sistem bio-pengimejan.

DEVELOPMENT OF A SILVER NANOCLUSTER PROBE AGAINST FOXP3 FOR LIVE TREGS SORTING : USING MCF-7 AS A MODEL

ABSTRACT

DNA-templated silver nanocluster (AgNC), a new promising fluorescence probe has been gaining importance in recent years. Owing to their special properties such as small in size, photostable and water-soluble, these fluorescence probes are favourable for many diagnostic applications. Due to these advantages, these AgNCs were adapted to develop a method for the identification of regulatory T cells (Tregs) through an intracellular transcription factor called forkhead box P3 (Foxp3). Tregs are crucial mediators of immune homeostasis, exerting suppression on effector cells in autoimmune diseases and promoting the cancer growth. Isolation of live Tregs is required for better characterisation. Current isolation of Tregs relies on a multitude of surface markers and a crucial intracellular marker, Foxp3. Isolation of a pure population is currently impossible as intracellular detection of Foxp3 relies on fixation and permeabilisation of cells, which often leads to cell death. Here, an optimised method for the detection of messenger ribonucleic acid (mRNA) of Foxp3 was introduced by hybridising AgNC and G-rich to its target. The hybridised samples were examined under UV illuminator and fluorescence intensity was determined by fluorescence spectroscopy. The successful hybridisation of a three-way junction with AgNC, G-rich and DNA/RNA Foxp3 target, produced an improved fluorescence intensity with spectral shift. The AgNC and GR-rich was successfully delivered into MCF-7 cells (a Foxp3 model), producing red fluorescence images which was corroborated by flow cytometry results. In summary, the silver nanocluster approach has the potential to detect intracellular Foxp3 nucleic acid to establish a non-lethal intracellular detection system for bioimaging.

CHAPTER 1 INTRODUCTION

1.1 Background of Study

Molecular and fluorescence probes are valuable tools in research as they provide a detection method that allows analysis at the cellular level. The two main types of probes often used in the cellular analysis are surface probes and intracellular probes. Both types of probe allow detection of cells with fluorescence assisted cell sorting (FACS). These cells can be further characterised and analysed for functional studies using surface markers. Intracellular probes, on the other hand, require permeabilisation of cells, which often leads to cell death. One of the application is the identification of regulatory T cells (Tregs).

Tregs are a subset of T cells specialised in maintaining the immune self-tolerance and homeostasis (Sakaguchi et al., 2008). It also plays a crucial role in avoiding autoimmunity as it acts as an immune suppressor (Lan et al., 2012). Studies have shown that the disruption in the development or function of Tregs can cause autoimmune diseases in mouse model and human (Sakaguchi et al., 2008). Tregs can be detected at a single cell level (Hori et al., 2003) using flow cytometry with the phenotype of $CD4^{+}CD25^{high}CD127^{-}Foxp3^{+}$ (Liu et al., 2006). Forkhead box P3 (Foxp3) is an important marker for Tregs, which is only found intracellularly. It is utilised for the identification and characterisation of Tregs. The expression levels of this marker are higher in Tregs (both resting or activated state) compared to the $CD4^{+}$ T helper cells (Birzele et al., 2011). A major drawback of the previous phenotype identification of Tregs is the necessity of cellular fixation and permeabilisation to detect intracellular Foxp3. The permeabilisation process creates pore on the cell membrane to allow the anti-Foxp3 antibodies enter the cell walls, which often causes

cell death (O'Brien and Bolton, 1995). Therefore, live Tregs are unable to be isolated for characterisation and functional studies.

This study focused on the development of silver nanoclusters probe for intracellular detection of Foxp3. This is to develop a method to identify and isolate Tregs in combination with other membrane-bound markers for single cell analysis. The nanocluster probe is designed according to probe design basis introduced by Yeh et al., 2010. The probe was designed as a complementary to the target strand (mRNA of Foxp3). The hybridisation of AgNC and G-rich in the presence and absence of Foxp3 target is illustrated in Figure 1.1. The probes are then delivered into cells with transfection reagent and analysed using fluorescence microscopy and flow cytometry.

1.2 Problem Statement

Tregs play a vital role in our immune system especially in maintaining immune homeostasis. To thoroughly study these cells, identification and isolation of these cells are required. However, this is hampered by the detection of one of its essential intracellular markers, Foxp3. Fixation and permeabilisation are required for intracellular identification of Foxp3. The fixed and sorted cells obtained are often not viable and the functionality of these pure populations of cells is impossible to characterise. Thus, molecular probes enabling intracellular live staining of Foxp3 could contribute to the first step of studying pure population of these cells in an isolated manner. Therefore, this study aimed to develop an approach utilising silver nanocluster probes that would allow live cell staining.

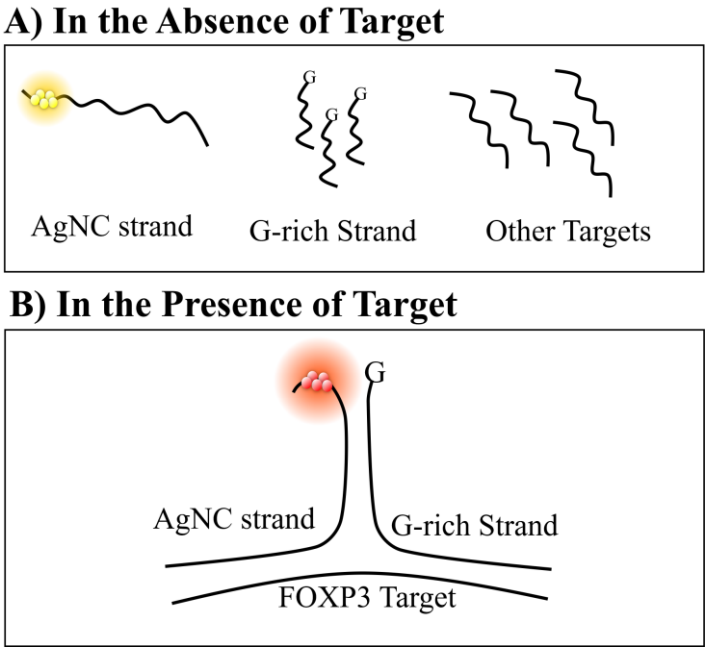

Figure 1.1 Hybridisation of AgNC and G-rich to the Foxp3 target when A) the target sequence is absence and B) the target sequence is available. In the absence of target, the three-way junction is not formed and no shift in fluorescence is observed. With the presence of target, the three-junction was form and a shift in fluorescence for AgNC is observed.

1.3 Research Objective

1.3.1 General Objective

- To develop nanocluster probe that enables intracellular live cell staining.

1.3.2 Specific Objectives

- To develop nanocluster probes to be delivered intracellularly
- To develop nanocluster probes that fluoresce upon binding to Foxp3 DNA target
- To develop nanocluster probes that fluoresce upon binding with its Foxp3 RNA target intracellularly

1.4 Significance of Research

In recent years, extensive research has emphasised on the role of Tregs in autoimmune diseases and cancers. This raises the importance of characterising and understanding the functionality of Tregs. However, accurate isolation of Tregs from heterogenous population of cells for single cell analysis would require intracellular staining of Foxp3, which remains a challenge. With this specific nanocluster probe capable of binding to Foxp3 DNA/RNA, live isolation of Tregs using flow cytometry can be made possible. This study explores the potential of AgNC to replace the conventional anti-Foxp3 antibody as the intracellular staining marker for Tregs, allowing the live intracellular cell staining.

CHAPTER 2 LITERATURE REVIEW

2.1 Regulatory T cells

2.1.1 Classification of Regulatory T cells

Regulatory T cells (Tregs) are $CD4^{+}CD25^{+}Foxp3^{+}$ immune cells that help in maintaining immune homeostasis (Hori et al., 2003). Tregs are divided into thymic Treg (tTreg) cells and peripheral (pTreg) cells. These thymus-derived Tregs (tTreg) made up a major population of Tregs, which are able to recognise host self-antigen major histocompatibility complex (MHC) of antigen-presenting cells (APC) in the thymus. However, this tTregs merge between true thymic originated Tregs and Tregs induced in peripheral from the thymic origin (Liston and Gray, 2014). pTregs are derived from $Foxp3^{-}CD4^{+}$ T cells in the peripheral to $Foxp3^{+}CD4^{+}$ Tregs after stimulating by IL-10 and transforming growth factor beta (TGF-β) cytokines (Sekiya et al., 2016, Shevach and Thornton, 2014). Other $Foxp3^{-}$ Tregs subsets include the IL-10 secreting Type 1 Tregs (Tr1) (Zeng et al., 2015) and T helper 3 Tregs (Th3) (Tang and Bluestone, 2008). These pTregs display a high grade of plasticity in response to immune adaptation (Valzasina et al., 2006, Geiger and Tauro, 2012). Studies found both tTregs and pTregs worked at a similar degree. However, best results in suppressing autoimmune diseases were achieved when both tTregs and pTregs present together (Haribhai et al., 2011, Huang et al., 2014). Induced Tregs (iTregs) are another group of Tregs cells stimulated from naïve T cells and those induced in *in-vitro* experiments (Curotto de Lafaille and Lafaille, 2009).

Besides the classification as mentioned above, Tregs are also proposed to be classified into other three groups, namely central Tregs, effector Tregs and tissue-resident Tregs (Liston and Gray, 2014). Central Tregs are the major population of Tregs circulating in the blood system and secondary lymphoid organs. These cells are

identified with CD45RA^{hi}CD25^{low}Tregs (Miyara et al., 2009). For effector Tregs, these cells are minority Tregs, which migrate through non-lymphoid tissues. Tissue-resident Tregs are referred as Tregs with long-term residency in non-lymphoid tissues. Each organ has a different population of tissue-resident Tregs depending on the functions and local immune regulation (Liston and Gray, 2014).

2.1.2 Identification of Regulatory T cells

Molecular markers are used to identify and distinguish cells. In order to thoroughly study Tregs, understanding of surface and intracellular markers are required for flow cytometry analysis (Figure 2.1). To characterise Tregs, highly expressed surface markers such as IL-2 receptor α chain (CD25) is used (de la Rosa et al., 2004). However, expression of CD25 is transiently up-regulated on activated effector T cells (Teff), which will not result in a pure population of Tregs if Tregs are isolated with this marker (Schmetterer et al., 2012). Therefore, additional markers are required for the identification of Tregs. One such marker is the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), the structural homologue of CD28. It acts as a negative regulator of T cell activation and binds to the similar ligand as CD28 which is the CD80 (B7-1)/ CD86 (B7-2) expressed on APC (Read et al., 2000).

Glucocorticoid-induced tumour necrosis factor (TNF) receptor family gene (GITR) is expressed on Tregs as well as resting CD4$^+$ and CD8$^+$ T cells with a lower level of expression (Shimizu et al., 2002, Durham et al., 2017). GITR are members of the TNFR superfamily (TNFRSF) which involve in the development of tTregs together with OX40 and TNFR2 (Mahmud et al., 2014). GITR functions to expand Tregs but do not take part in the suppressive function of Tregs (Ronchetti et al., 2015). GITR is activated by its ligand (glucocorticoid-induced tumour necrosis factor (TNF) receptor

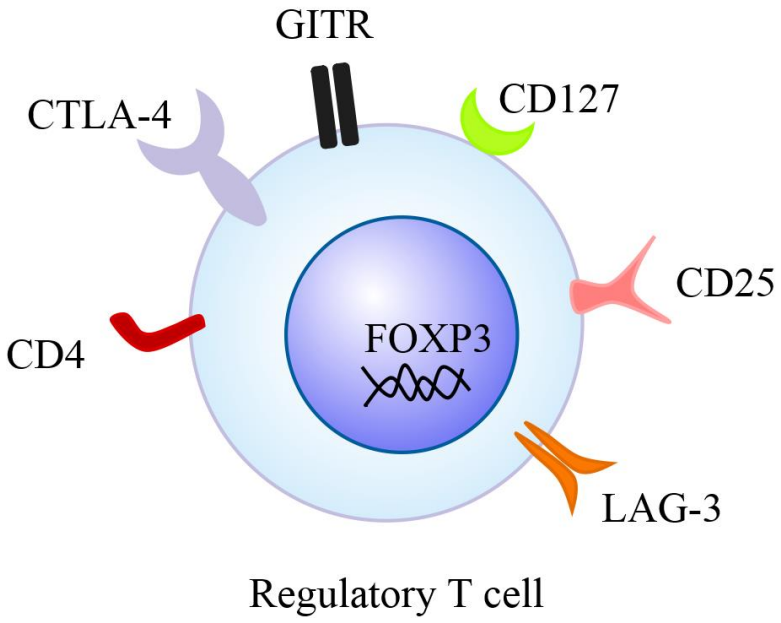

Figure 2.1 Surface markers and intracellular markers of regulatory T cells.

family gene ligand (GITRL)), expressed by activated APCs and endothelial cells. The co-stimulation of GITR increases IL-10 production (Nocentini et al., 2008).

TGF-β latency-associated peptide (LAP) is a homodimer of a propeptide that forms a latent TGF-β complex that promotes the conversion of naïve Tregs to iTregs and mediates Tregs-associated immune suppression (Gandhi et al., 2010). LAP is expressed on the cell membrane of many immune cells and participates in immune regulation. Studies have shown that LAP-positive Tregs have better suppressive functions compared to LAP-negative Tregs. (Mahalingam et al., 2014)

Foxp3 is an important marker of Tregs, constitutively expressed in tTregs cells to exhibit its suppression function. Foxp3 is a family of forkhead box (FOX) transcription factors encoded on the X chromosome characterised by a highly conserved forkhead DNA-binding domain. Studies found mutation of Foxp3 has resulted in immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (Bennett et al., 2001). Structurally, Foxp3 consists of a leucine zipper-like domain and zinc finger motif. The N terminus domain is thought to be the repressor domain (Li and Greene, 2007). Foxp3 expression is not limited to Tregs cells but could also be expressed by epithelial cells. Expression in epithelial cells may act as tumour suppressor gene in certain cancers such as breast and prostate cancer. Foxp3 expression was found in normal epithelial cells, however, its downregulation was related to cancer development (Chen et al., 2008, Zuo et al., 2007). Controversially, other studies also indicated that Foxp3 is involved in cancer biology as they demonstrated that culturing of Foxp3-expressing pancreatic carcinoma cells with naïve T cells can inhibit the proliferation of T cells (Hinz et al., 2007). This proposes that expression of Foxp3 in different tumour cells may function differently. Studies

by Karanikas et al. have shown high Foxp3 expression in MCF-7 cells besides Tregs (Figure 2.2).

Several other transcription factors besides Foxp3, are also identified in Tregs. $Foxp3^-$ Tregs was also reported to exhibit the function of Tregs with others identified markers such as Helios, Neuropilin 1(Nrp-1) and LAP/GARP (Himmel et al., 2013).

Helios and Nrp-1 are suggested as markers to differentiate tTregs and pTregs (Thornton et al., 2010, Weiss et al., 2012). There are many controversies regarding these two markers. Helios, Ikaros zinc finger transcription factor is a selective marker for mice tTreg and identified in pTregs, tTregs, $CD8^+$ T cells, activated T cells and T cells in inflammatory environments (Akimova et al., 2011, Singh et al., 2015). Helios is proposed to be a specific marker for tTregs but not for pTregs (Thornton et al., 2010). However, findings in Himmel et al., 2013 study showed that Helios-negative tTregs have no significant difference in phenotype and function with Helios-positive tTregs, suggesting its inability to distinguish tTregs and pTregs (Himmel et al., 2013). Helios deficiency does not hamper the development of tTregs.

Neuropilin-1(Nrp-1), a semaphorin III receptor functions to promote angiogenesis, is found highly expressed on the surface of tTregs and proposed as a marker for tTreg under certain conditions (Milpied et al., 2009, Weiss et al., 2012). Nrp-1 expression helps to increase Tregs capacity to infiltrate tumours (Chaudhary et al., 2014) and improves Tregs stability at inflammatory sites by supporting the differentiation of naive $CD4^+$ cells to pTregs and interferes with their differentiation to T helper 17 (Th17) cell lineage (Szurek et al., 2015). However, among many suggested markers, none of the markers above can replace Foxp3 as Foxp3 deletion was showed to hamper the function of Tregs. This implies the importance of Foxp3 to identify Tregs cell.

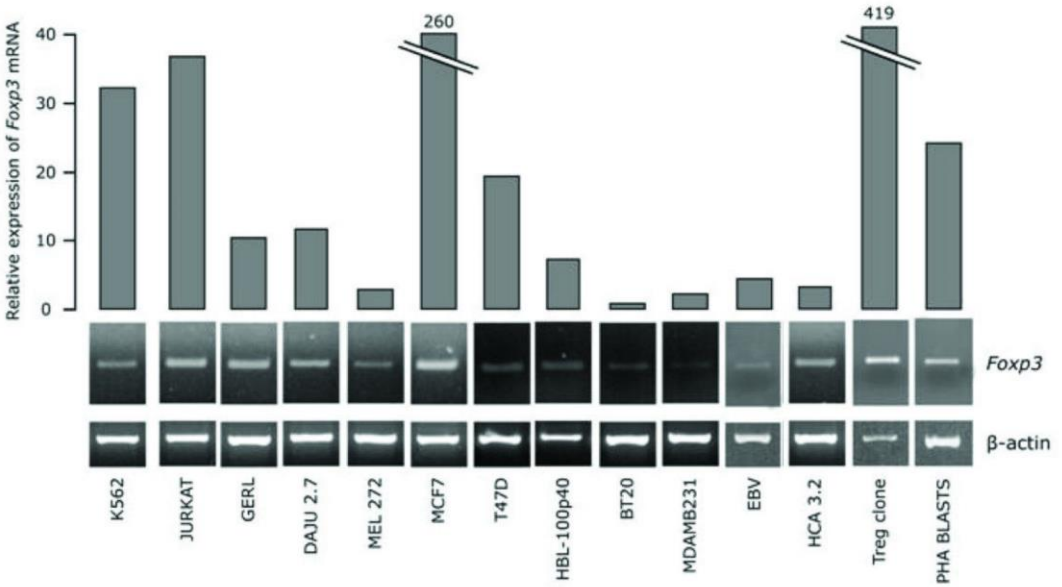

Figure 2.2 Foxp3 mRNA expression in tumour cells lines using qRT-PCR and RT-PCR. The level of Foxp3 expression (qRT-PCR) in MCF-7 was the second highest after Tregs (Karanikas et al., 2008)

2.1.3 Mechanism of Suppression by Regulatory T cells

Tregs suppresses many immune cells such as T cells, B cells and APC (Chen et al., 2008, Misra et al., 2004). The suppressive functions of Tregs are divided into four different modes which are mediated by modulating APC maturation and function (Zheng et al., 2004), killing of target cell (Grossman et al., 2004), disruption of metabolic processes (Yan et al., 2010) and production of anti-inflammatory cytokines (Vignali et al., 2008). For modulation of APC maturation and function, CD80 and CD86 play a very important role as co-stimulatory molecules, binding to CD28 and CTLA-4 of Tregs subset. The binding of CTLA-4 to CD80 or CD86 leads to the down-regulation of CD80 or CD86 mRNA, which downregulates the CD80 or CD86 expression. Therefore, it limits the ability of APC to initiate an adaptive immune response. CTLA-4 together with LAG-3 facilitates the interactions between Tregs and dendritic cells (DC) in inhibiting the maturation of DC that limits antigen presentation and activation of T effector cells (Liu et al., 2016).

In mice and humans, the mechanism of killing effector cells by Tregs is achieved via the expression T cell apoptosis-inducing molecules such as granzymes (A and B) and perforin (Grossman et al., 2004). Granzymes are enzymes released from cytolytic granules which enter target cells through perforin pores and activate the caspase pathway and apoptotic effector cell death (Vignali et al., 2008).

Metabolic disruption involves IL-2 deprivation of Tregs, where IL-2 is an indispensable cytokine for the proliferation of effector T cells. CD39 is an ectonucleotidase expressed constitutively by murine Tregs. It catalyses the degradation of adenosine triphosphate and produces adenosine to suppress Teff. Adenosine bound to the adenosine receptor 2A promotes TGF-β expression and inhibits IL-6 expression. IL-6 inhibition leads to intracellular cyclic adenosine

monophosphate (cAMP) transport to Teff through gap junction, resulting in inhibition of nuclear factor of activated T-cells (NFAT) and IL-2 transcription. Therefore, its inhibition leads to cell apoptosis by IL-2 deprivation (Vignali et al., 2008, Safinia et al., 2015).

Tregs also produce anti-inflammatory cytokines such as TGF-β, IL-10 and IL-35. TGF-β plays a pivotal role in differentiating and maintaining the function of tTregs (Chen and Konkel, 2010). IL-10 inhibits the activation of macrophages and dendritic cells (Schmetterer et al., 2012). These mechanisms play an important role in maintaining the immune kinetics but could also lead to the emergence of diseases such as cancer.

2.2 Silver Nanocluster

2.2.1 Properties of Silver Nanocluster

Silver nanocluster was first introduced in the year 2002 by the Dickson group where they created an easily observed in single molecular level of silver encapsulated nanoclusters with dendrimer in aqueous solution (Zheng and Dickson, 2002). Since then, many applications involving silver nanocluster have emerged in various detection methods in chemical and biological fields. Silver nanocluster can be synthesised through two methods, which is by reducing the silver ions with a reducing agent (sodium borohydride, sodium citrate and sodium hypophosphite) or by light where the visible or ultraviolet light is used (Diez and Ras, 2011). However, sodium borohydride is commonly used to reduce silver ions because it is a strong reducing agent for metal ions such as silver (Mavani and Shah, 2013, Brown and Rao, 1956). As for weaker reducing agent such as sodium citrate (Wani et al, 2010), it produces smaller silver nanoparticles with a smaller size distribution (Wani et al., 2011). To

increase the stability of silver ions, different stabilisers such as polymers, DNA oligonucleotides and dendrimers are utilised (Zheng and Dickson, 2002, Latorre et al., 2013, Shang and Dong, 2008). The size of AgNC with less than 2 nm can be controlled by altering the ratio of stabilizer and Ag (Hua and Hongtao, 2015). DNA has a high binding affinity for metal cations such as Mg^{2+}, Au^{+} and Ag^{+} ions. The Ag^{+} ions bind covalently to DNA through nucleobases especially cytosine and guanine at N7 and O6 (Obliosca et al., 2013b, Petty et al., 2004). These Ag^{+} ions can be reduced to form metallic nanoparticles as a template (Petty et al., 2004).

One of the advantages of using AgNC is the homogeneous (in solution) detection of specific DNA or RNA that precludes washing steps. Since unbound probes are not removed, therefore low background fluorescence silver nanocluster displays a unique signature of a shift in fluorescence after hybridising to a specific target sequence (Obliosca et al., 2013a).

2.2.2 Factors affecting silver nanocluster

The physical colour of silver nanocluster is affected by various factors including the number of silver ions that binds to the DNA complexes, pH or types of buffers utilised in the synthesis. The base sequence of single-stranded DNA (ssDNA) template to stabilise the AgNC is also a considering factor in designing the AgNC.

2.2.2(a) Binding of Silver Ions

The number of silver ions binding to oligonucleotides affects the fluorescence colour of the synthesised silver nanoclusters. In the study by Copp et al., 2014, the random rapid array screening summarised with the binding of four silver ions, green fluorescence AgNCs are usually detected at emission range of 540 nm. Red

fluorescence AgNCs are often detected by the binding of six silver ions. An addition or removal of a single Ag atom/ion can increase or decrease the peak emission wavelength of an AgNC by approximately 20 nm. Therefore, smaller and more negatively charged clusters left shifts to the blue visible region while larger and more positively charged clusters are right shifted to the red region (Schultz and Gwinn, 2012).

2.2.2(b) DNA sequence of Silver Nanocluster Template

Different sequences of DNA templates affect the emission wavelength of AgNC (Table 2.1). Cytosine-rich template produces green emissive AgNC at 520 nm while the combination of cytosine and thymine bases produces blue emissive AgNC at 475 nm (Latorre and Somoza, 2012, Choi et al., 2011).

DNA hairpin sequences determine different types of fluorescent clusters (O'Neill et al., 2009). Studies by Shah et al., 2014 demonstrated the secondary structure of DNA/AgNCs designs were one of the factors affecting fluorescence intensity of AgNC. The highest binding affinity of DNA structure contributes to the stability of AgNC was the coiled C-rich strand, followed by i-motif, duplex and G-quadruplex (Li et al., 2013). All these factors must be taken into consideration in designing AgNC to ensure a stable AgNC for detection.

2.2.2(c) Effects of Buffer on Silver Nanocluster

Buffers are important in maintaining the pH of the solution and helps in nucleic acid hybridisation. Tris-Cl buffers provide monovalent cation, $TrisH^+$ to anneal two strands of nucleic acids (Shah et al., 2014). However, the concentration of chloride ions in the Tris-Cl buffer can turn off silver ions emission by solubilisation or precipitation of

Table 2.1 Excitation and Emission Wavelength of Different DNA Template Sequence

Sequence	**Excitation/Emission Wavelength (nm)**	**References**
Blue		
5’ TTTTCCCCTTTT 3’	370/475	(Latorre and Somoza, 2012)
5’CCCTTTAACCCC 3’	340/485	
Green		
5’ CCCTCTTAACCC 3’	425/520	(Richards et al., 2008)
5’ C8TTAATC8 3’	460/555	(Obliosca et al., 2014)
Yellow		
5’ AATTCCCCCCCCCCCC 3’	480/562	(Choi et al., 2011)
Red		
5’ (GGGTAA)3GGGTA 3’	560/626	(Liu et al., 2013)

AgCl. Therefore, all anionic buffers are not recommended for use in synthesising AgNC as anionic ions bind to silver ions and disable its fluorescence. To replace chlorine ions, Tris-acetate buffer is used as an alternative. However, the emission intensity of the probe gradually decreases as the concentration of Tris-acetate buffer increases. Although the emission intensity for Tris-acetate buffer has a 30 % decrease, the emission intensity is better than in Tris-Cl buffer. Potassium nitrate and sodium nitrate buffers provide higher emission intensity as compared to water in the preparation of silver nanocluster. Magnesium sulphate, magnesium acetate and magnesium nitrate impeded the emissive silver nanocluster. (Shah et al., 2014) Study by Petty et al. showed that sodium ions are important for DNA target recognition by the AgNCs (Petty et al., 2004). In this study, sodium phosphate buffer was used as a buffer to increase the hybridisation efficiency of AgNC.

2.3 Transfection

As nucleic acids are negatively charged, a carrier is required to facilitates the crossing through the hydrophobic cellular membrane. The delivery of nucleic acids into cells can be achieved by transfection (Reed et al., 2005). Transfection is commonly used to deliver foreign genes and plasmids into cells for protein overexpression purposes (Wurm, 2004). There are two types of transfection methods being used – conventional (Forward) and reverse (Rev) transfection (Yamada et al., 2009, Antoku et al., 2010). The conventional transfection is commonly used for adherent cells where the cells are plated a day earlier before the incubation of the transfection complexes. It is divided into three methods – chemical, physical and viral methods (Kaestner et al., 2015). Reverse transfection, on the other hand, is conducted with pre-plated transfection complexes and freshly subcultured cells, allowing direct and sufficient contact

between cells and transfection complexes, thereby improving transfection efficiency (Erfle et al., 2007).

The chemical transfection methods commonly used include calcium phosphates, cationic liposomes and polyethyleneimine (PEI) (Pandey and Sawant, 2016, Hoekstra, 2001, Kingston et al., 2001). Chemical transfection works by coupling of the positively-charged chemicals with negatively-charged nucleic acids to form an overall positively-charged nucleic/chemical complexes. These positively charged complexes can then be delivered across the negatively charged cell membrane. Physical methods of transfection include microinjection and electroporation (Lim et al., 2010, Chu et al., 1987). Viral methods allow stable cell lines transfection using viral vectors such as lentiviral (Tiscornia et al., 2003), adenoviral (Becker et al., 1994) and retroviral vector (Miller and Rosman, 1989).

Calcium phosphate transfection, established by Graham and Van in 1973 is often used to generate cell lines for transient expression of proteins (Graham and van der Eb, 1973). However, there are several drawbacks of using calcium phosphate including its toxicity, especially towards primary cells, sensitive to slight changes in pH, temperature and buffers salt concentrations as well as its poor transfection efficiency as compared to other chemical transfection methods such as lipofection (Jordan et al., 1996).

Cationic lipid (liposomes) are amphiphilic molecules with hydrophilic and hydrophobic region that can enter the cells via an endocytotic pathway (Xu and Szoka, 1996). The liposomes or cationic lipids with a charged head group and one or two hydrocarbon chains can interact with the phosphate backbone of DNA and facilitates DNA condensation (Wasungu and Hoekstra, 2006). This interaction of liposomes allows fusion of DNA with the negatively charged cell membrane and enters the cells

through endocytosis (Wrobel and Collins, 1995). Endocytosis is a process where a localised region of the cellular membrane uptaking the DNA-liposome complex by forming a membrane-bound/intracellular vesicle (Chesnoy and Huang, 2000).

2.4 Current Trend of Live Fluorescence RNA Detection

SmartFlare probe is a recent gold nanoparticles probe commercially available from Merck, covalently labelled with multiple capture oligonucleotides specific to a protein of interest (McClellan et al., 2015). It allows live fluorescence detection of mRNA in cells. The probe comprises of a reporter flare sequence that hybridised to the recognition sequence with a cyanine3 or cyanine 5 fluorophore (Figure 2.3). The fluorophore dye is quenched when it is in close proximity to the gold surface. When the reporter sequence is displaced by complementary mRNA, the free flare sequence provides a fluorescent signal (Halo et al., 2014). This fluorescence signal can be used for fluorescence microscopy, flow cytometry and FACS.

However, there is only 21 targets available to be purchased. Despite the similarity in SmartFlare's objective to enable live cell staining, there are several reasons why silver nanoclusters were still preferred in this study. Silver nanoclusters are more cost effective to prepare and to be applied for detecting a variety of other targets as it would only require synthesised oligonucleotides. This would also mean that the adaptability of the silver nanocluster provides ease in the detection of other necessary targets. Aside from that, the three-way junction design of silver nanocluster allows the detection of up to a single nucleotide polymorphism (SNP) (Yeh et al., 2012) enabling a more sensitive detection of mRNA in cells.

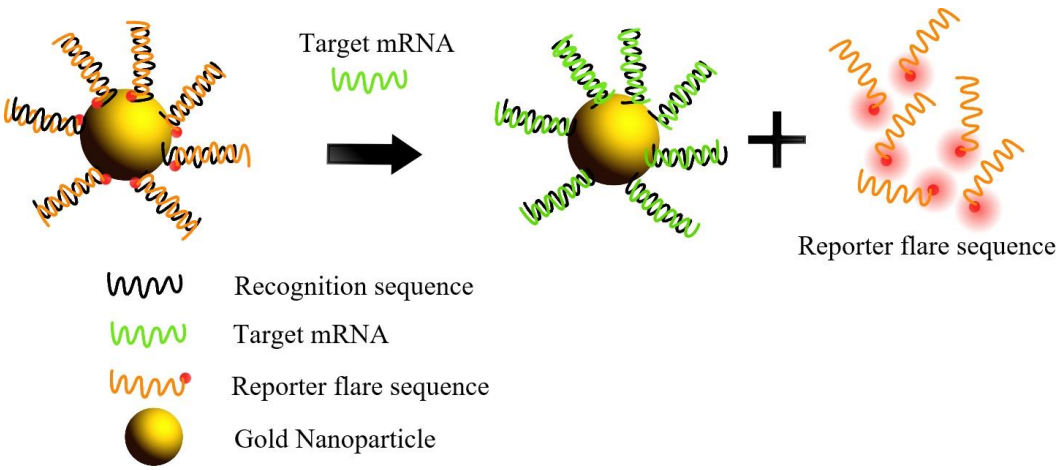

Figure 2.3 Structure of SmartFlare. The SmartFlare probe comprises of multiple DNA oligonucleotide sequences covalently bound to the 13-nm surface of the spherical gold nanoparticle. A reporter flare sequence labelled with Cyanine 5 (red) is quenched when hybridises close proximity to the target recognition sequence. When the sequence is displaced by target mRNA (green), the reporter flare sequence produces fluorescence signal for detection.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The chemicals used in this study are listed in Table 3.1.

3.1.2 Molecular Reagent

The reagent used for molecular work are listed in Table 3.2

3.1.3 Reagent for Cell Culture

The reagent used for cell culture are listed in Table 3.3.

3.1.4 Consumable Items

The consumable items used in this study are listed in Table 3.4.

3.1.5 Apparatus and Instruments

All apparatus and instruments used throughout this study are listed in Table 3.5.

3.1.6 Computer Softwares

The computer softwares used in this study are listed in Table 3.6.

3.1.7 Oligonucleotides

The sequences of oligonucleotides used in this study are listed in Table 3.7.

Table 3.1 List of chemical reagents

Name	Manufacturer	Location of Origin
Acrylamide	Merck	Darmstadt, Germany
Ammonium persulfate (APS)	Promega	Madison, USA
Ampicillin sodium salt	Nacalai Tesque	Japan
Boric Acid	Merck	Darmstadt, Germany
Bis-acrylamide	Promega	Madison, USA
Disodium hydrogen phosphate	Merck	Darmstadt, Germany
Ethidium bromide	Amresco	Ohio,USA
Ethylenediaminetetraacetic acid (EDTA)	Merck	Darmstadt, Germany
Glycerol	Merck	Darmstadt, Germany
Hydrochloric acid fuming 37%	Merck	Darmstadt, Germany
Isopropanol	Merck	Darmstadt, Germany
Potassium Chloride	Merck	Darmstadt, Germany
Potassium dihydrogen phosphate	Merck	Darmstadt, Germany
Silver nitrate	Merck	Darmstadt, Germany
Sodium borohydride	Merck	Darmstadt, Germany
Sodium Chloride	Merck	Darmstadt, Germany
Sodium dihydrogen phosphate	Merck	Darmstadt, Germany
Tetramethylethylenediamine (TEMED)	Merck	Darmstadt, Germany
Tris-HCl	First Base	Selangor, Malaysia

Table 3.2 Molecular Reagents

Name	Manufacturer	Location of Origin
6x DNA Loading Dye	Thermo Fisher Scientific	Massachusetts,USA
Agarose powder	Firstbase	Selangor, Malaysia
dNTPs	Thermo Fisher Scientific	Massachusetts,USA
Q5 High Fidelity DNA Polymerase	New England Biolabs	Ipswich, MA
RNeasy Mini Kit	Qiagen	Germany
QIAquick Gel Extraction Kit	Qiagen	Germany
Tetro cDNA Synthesis Kit	Bioline	Taunton, MA

Table 3.3 Reagents for cell culture

Name	Manufacturer	Location of Origin
Dulbecco's Minimum Essential Medium (DMEM)	Nacalai Tesque	Japan
Dimethyl sulfoxide (DMSO)	Nacalai Tesque	Japan
Fetal Bovine Serum (FBS)	Nacalai Tesque	Japan
Lipofectamine 2000	Invitrogen	Massachusetts,USA
Penicillin/Streptomycin	Nacalai Tesque	Japan
Trypsin-EDTA	Nacalai Tesque	Japan
Trypan Blue Solution	Nacalai Tesque	Japan

Table 3.4 Consumable items

Name	Manufacturer	Location of Origin
0.2 mL PCR Strips	Axygen	Massachusetts,USA
1.5 mL Microcentrifuge tube	Ratiolab	Germany
6 well plates	Thermo Fisher Scientific	Massachusetts,USA
20 gauge syringe needle		
24 well plates	Thermo Fisher Scientific	Massachusetts,USA
15 mL Centrifuge tube	SPL	Korea
50 mL Centrifuge tube	SPL	Korea
Flask (T25 and T75)	Thermo Fisher Scientific	Massachusetts,USA
Glasswares	Duran	Germany
Pipette Tips	Gilson	Wisconsin, USA
10 mL and 25 mL serological pipette	Thermo Fisher Scientific	Massachusetts,USA

Table 3.5 Apparatus and instrument

Name	Manufacturer	Location of Origin
Autoclave	Hirayama	Saitama, Japan
Balance	Mettler Toledo	Greifensee, Switzerland
Centrifuge	Eppendorf	Hamburg, Germany
Fluorescence Microscope	Zeiss Axio Observer A1	Carl Zeiss, Germany
Fluorescence Reader Cary Eclipse	Varian	California, USA
FACSVerse	BD Biosciences	USA
Freezer (-20°C)	Thermo Fisher Scientific	Massachusetts,USA
Ice maker	Thermo Fisher Scientific	Massachusetts,USA
Incubator	Thermo Fisher Scientific	Massachusetts,USA
Inverted microscope	Olympus	Tokyo, Japan
Multiskan Spectrum	Thermo Fisher Scientific	Massachusetts,USA
pH meter	Thermo Fisher Scientific	Massachusetts,USA
Power Pack	Bio-Rad	California, USA
Refrigerator	Thermo Fisher Scientific	Massachusetts,USA
Thermal Cycler	Bio-rad	California, USA
Thermomixer	Eppendorf	Hamburg, Germany
UV Illuminator	Syngene	UK
UV-Vis Spectroscopy	Agilent Technologies	Germany
Vortex Mixer	Thermo Fisher Scientific	Massachusetts,USA

Table 3.6 Computer software

Software	**Application**	**Developer**	**Web Page**
Adobe Acrobat DC	For file viewing and editing in Portable Document Format (PDF)	Adobe System Inc.	https://get.adobe.com/reader/?loc=uk
GraphPad Prism 6	For data and graph analysing, editing and viewing	GraphPad Software, Inc.	http://www.graphpad.com/prism/prism.htm
Microsoft Office 365	For word processing, spreadsheets, presentations	Microsoft Corp.	http://office.microsoft.com/
Flowjo VX	For flow cytometer results analysis	Flowjo, LLC	https://www.flowjo.com/

Table 3.7 Oligonucleotides sequence

Name	Sequence	Reference / Comments
Silver Nanocluster (AgNC) Strand		
NC2	5' CCC TTA ATC CCC ATA CAG CTG CAG CTG CGA 3'	(Yeh et al., 2012)
NC2_B	5' CCC TTT AAC CCC ATA CAG CTG CAG CTG CGA 3'	(Richards et al., 2008)
NC2_R	5' CCT CCT TCC TCC ATA CAG CTG CAG CTG CGA 3'	(Richards et al., 2008)
20C	5'CCC CCC CCC CCC CCC CCC CCA TAC AGC TGC AGC TGC GA 3'	(Sharma et al., 2010)
24C	5'CCC CCC CCC CCC CCC CCC CCC CCC ATA CAG CTG CAG CTG CGA 3'	(Sharma et al., 2010)
NC_end	5' CAG CTG CAG CTG CGA 3'	
20CR	5' CCC CCC CCC CCC CCC CCC CCC CCC ATA CAG CTG CAG CTG CGA TGG TGG CAT GGG GTT CAA GGA AGA AGA GGA GGC AT 3'	
G-rich and Foxp3 Strand		
G-rich	5' GAC TAG GGG CAG TGT GGG TAT GGG TGG GGT GGG GTG GGG 3'	(Yeh et al., 2012)
GR-rich	5' GGG TGC CAC CAT GAC TAG GGG CAG TGT GGG TAT GGG TGG GGT GGG GTG GGG 3'	
Foxp3 Target	5' TCG CAG CTG CAG CTG CCC ACA CTG CCC CTA GTC 3'	
Non-specific Target	5' CTT CCT CAA GCA CTG CCA GGC GGA CCA TCT 3'	
PCR Primers		
Foxp3 Forward	5' GCC CTT GGA CAA GGA CCC GAT G 3'	
Foxp3 Reverse	5' CAT TTG CCA GCA GTG GGT AGG A-3'	
CYPA Forward	5' GTC AGC AAT GGT GAT CTT CTT 3'	
CYPA Reverse	5' GCA GAA AAT TTT CGT GCT CTG 3'	