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**Smart Drug Delivery Systems for siRNA Therapeutics in
Prostate Cancer Immunotherapy**

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Abstract

Prostate cancer (PCa) as an immunosuppressive cancer remains a serious condition threatening the health of men due to the complicated nature of the tumour microenvironment (TME). Conventional treatments of PCa face challenges including poor prognosis and tumour resistance, therefore new therapeutic strategies are urgently needed. Tumour-associated macrophages (TAMs) are a potential therapeutic target in cancer immunotherapy. Colony stimulating factor-1/colony stimulating factor-1 receptor (CSF-1/CSF-1R) pathway plays a crucial role in the polarization of the immunosuppressive TAMs, M2 macrophages. Downregulation of CSF-1R expression by small interfering RNA (siRNA), a double-stranded non-coding RNA, is known to reprogram the immunosuppressive TAMs, M2 macrophages, to the immunostimulatory phenotype, M1 macrophages. Sialic acid (SA) is a ligand for Siglec-1 (CD169) which is overexpressed on M2 macrophages with little expression in other phenotypes. M2 macrophage-targeting peptide (M2pep) is an artificially designed peptide and could specifically target M2 macrophages, the sequence of which was selected by subtractive phage biopanning. Beta-cyclodextrins (CDs) are cyclic oligosaccharides derived from starch, consisting of at 7 glucose subunits linked by α -1,4 glycosidic bonds, the primary and secondary sides of the ring structure are hydrophilic and the cavity is hydrophobic. Cationic CDs have been used for delivery of therapeutic nucleic acids including siRNA as non-viral vectors. Therefore, in this thesis, 2 different

CD-based nanoparticles (NPs) with PEGylated SA or PEGylated M2pep as the ligand was designed and characterized for the delivery of CSF-1R to M2 macrophages in both human and mouse cells to study the macrophage reprogramming efficacy. In the meanwhile, a Transwell model was built for co-culture of M2 macrophages and PCa cells to test the anti-PCa effectiveness *in vitro*. *In vitro* results show that the sizes of three NPs were around 250 nm, and the surface charges were 11 ~ 29 mV, the poly dispersity index (PDI) was lower than 0.30. All CD-based formulations were stable in both salt- and serum-containing media, and did not present any cytotoxicity in either human or mouse macrophages. Both two NPs achieved M2 macrophage reprogramming, and post-transfected cells expressed M1 macrophage markers. The co-culture results show that the formulations caused the apoptosis of PCa cells due to the M2 macrophage reprogramming.

In the *in vitro* studies, the CD.siRNA.DSPE-PEG-M2pep formulation presented the best CSF-1R gene silencing efficacy, superior M2 macrophage reprogramming and anti-tumour efficacy. Therefore, the CD.siRNA.DSPE-PEG-M2pep formulation was selected for assessment *in vivo*. A PCa-bearing mouse model was built in C57 BL/6 mice by subcutaneous injection of PCa cells and used to study the targeting and anti-PCa efficacy of CD.siRNA.DSPE-PEG-M2pep *in vivo*. The NPs increased M2 macrophage targeting *in vivo*, promoting the release of M1 factors and simultaneously downregulating the levels of M2 factors through TAM

reprogramming. The subsequent remodelling of the TME resulted in a reduction in tumour growth in the PCa mouse model, mainly mediated through the recruitment of cytotoxic T cells.

In summary, the CD-based targeted siRNA NPs successfully reprogrammed the immunosuppressive M2 macrophages thus promoting PCa immunotherapy and providing an alternative strategy for PCa treatment worthy of further investigation.

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Chapter 1 Introduction

1.1 The Tumour Microenvironment and Tumour Associated Macrophages

1.1.1 Tumour Microenvironment

Solid tumours are surrounded by a complex environment called the tumour microenvironment (TME) (Figure 1.1). The composition of the TME includes; tumour cells, immune cells, fibroblasts, endothelial cells, and pericytes, signalling molecules; including chemokines, cytokines, and growth factors, the extracellular matrix (ECM), blood vessels, and acidic/hypoxic environments (Quail and Joyce, 2013). The dense ECM and blood vessels impede the delivery of therapeutic agents to tumour cells, and constitute barriers to drug delivery in cancer therapy (Dewhirst and Secomb, 2017). In addition, the hypoxic and acidic nature of the TME can inactive certain therapeutics leading to therapeutic failure (Lequeux et al., 2019).

1.1.2 Tumour-Associated Macrophages

Immune cells play important roles in the TME, specifically tumour-associated macrophages (TAMs), which make up around 50% of tumour tissues (Li et al., 2020). TAMs are associated with cancer growth and metastasis, and the existence of TAMs has been reported to be closely associated with poor prognosis in major cancer types, including prostate cancer, breast cancer, and melanoma (Fridman et al., 2017).

Macrophages and dendritic cells (DCs) are differentiated from monocytes and monocytic myeloid-derived suppressor cells (M-MDSCs), which are derived in the bone marrow (BM) via hematopoietic stem cells (HSCs) → granulocyte-macrophage progenitors (GMPs) → monocyte progenitors (MP) → monocytes/M-MDSCs (Weston et al., 2018). Generally, TAMs are categorized into 2 different phenotypes, M1 and M2 macrophages, M1 macrophages are immunostimulatory cells and regarded as an “anti-tumourigenic” cell type, while M2 macrophages are immunosuppressive cells, which can promote tumour growth and metastasis. Both M1 and M2 regulate the TME by secreting different factors to affect tumour progression. M1 factors include M1 cytokines like interleukin-12 (IL-12), and chemokines such as the C-X-C motif chemokine ligand 10 (CXCL-10), which can directly cause tumour reduction and can indirectly recruit CD8⁺ cytotoxic T cells into tumour areas which subsequently achieve tumour immunotherapy. In contrast, M2 factors like interleukin-6 (IL-6) and arginase-1 (Arg-1) initiate the opposite effect, they promote differentiation of TAMs into the M2 phenotype and are the main reason for drug resistance and suppression of immunotherapeutics (Pathria et al., 2019, Biswas and Mantovani, 2010). Therefore, TAMs are the key regulator of TME, and the polarization of TAMs is closely associated with tumour development and progression (Figure 1.1).

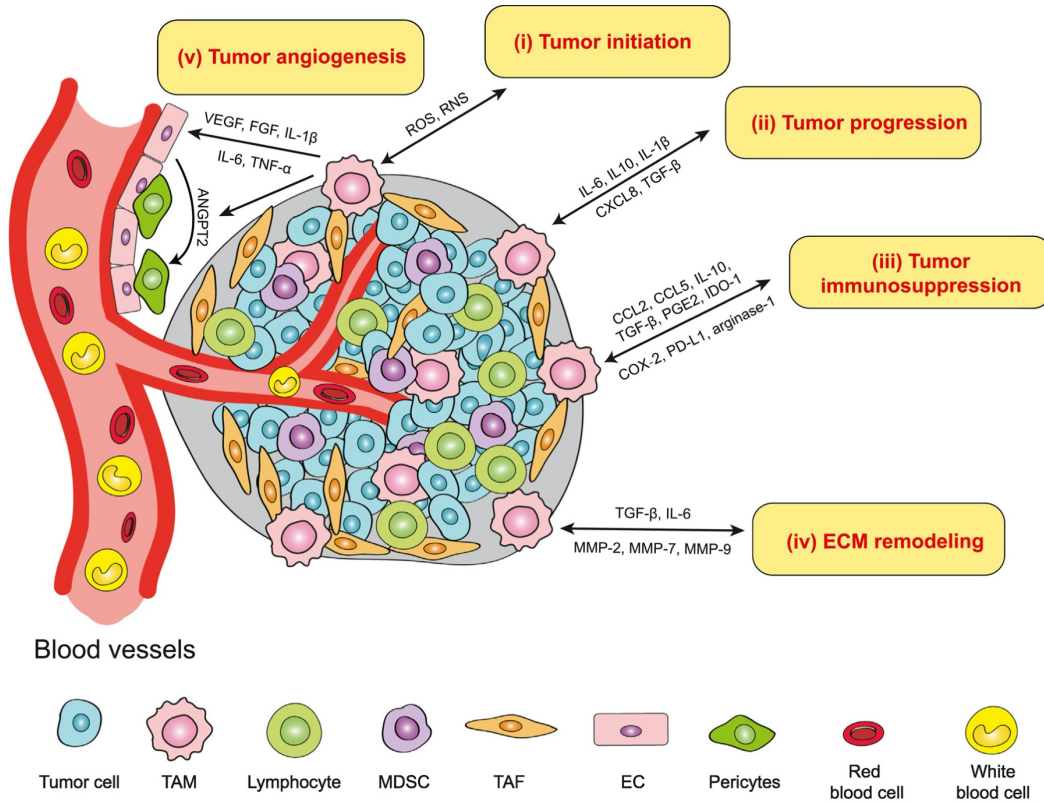


Figure 1.1 Tumour-associated macrophages (TAMs) are a key regulator of the tumour microenvironment (TME) (Yang et al., 2020).

1.1.3 TAMs and Tumour Growth, Development and Metastasis

In the early stage of cancer, immunosuppressive TAMs (M2 macrophages) release reactive oxygen species (ROS) and reactive nitrogen species (RNS) to enhance the genetic instability of pre-malignant cells (Elinav et al., 2013). Subsequently, in the developing stage of cancer, they promote tumour progression and angiogenesis via releasing soluble signalling molecules, such as interleukin-6 (IL-6), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and fibroblast growth factors (FGF) (Grivennikov et al., 2010).

At the same time, M2 macrophages recruit Myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs) and anti-inflammatory/tumour-promoting cells into the tumour areas and downregulate the level of pro-inflammatory cells, such as cytotoxic T cells via secreting M2 chemokines, including CCL2 (monocyte chemoattractant protein-1, MCP-1). These cells will in turn promote the differentiation of further M-MDSCs into M2 cells, forming a vicious circle. This leads to a strong immunosuppressive environment within the TME, which reduces the ability of conventional therapy, such as chemotherapy or radiotherapy, to achieve the expected anti-tumour effects (Togashi et al., 2019, Mantovani et al., 2017).

It is reported that fibroblasts in the tumour area also plays an important role in tumour growth, development, and metastasis, these fibroblasts are called tumour-associated fibroblasts (TAFs). TAFs and TAMs co-exist within the ECM, leading to tumour chemoresistance (Kalluri, 2016). The TAMs maintain the TAFs in the activate state through secretory factors, like TGF- β 、PDGF and FGF. The activated TAFs promote the formation of dense structures through desmoplasia within the tumours by upregulating the expression of collagen I (Chen and Song, 2019). This bi-directional TAM-TAF signalling pathway is also closely associated with the formation of blood vessels in tumours.

Hypoxia is one of the tumour hallmarks (Lewis et al., 2016), stimulated by hypoxia-inducible factors, like CXCL12. TAMs migrate to the tumour hypoxic site, and release

M2 factors promoting angiogenesis to provide oxygen for tumour cells (Yang et al., 2018). These M2 factors, including growth factors, like VEGF and FGF, and M2 cytokines, such as IL-6 and TNF- α , which activate vasculature formation through stimulating vascular cells (De Palma et al., 2017). At the same time, the TAMs promote lymphangiogenesis, which promotes tumour spread and metastasis by delivering tumour cells to regional lymph nodes (Lewis et al., 2016). It has also been reported that a hypoxic environment can in turn induce M2 polarization of TAMs thus forming a vicious cycle that promotes angiogenesis and tumour growth (Vitale et al., 2019).

1.2 Immunotherapy

As mentioned above, immunosuppressive TAMs are one of the main reasons for the clinical failure of conventional cancer treatment strategies. In recent years, research has focused on the development of new treatment strategies targeting TAMs. This novel immunotherapeutic strategy to regulate the immune system through TAMs is mainly divided into two categories: reducing M2 macrophage recruitment and survival in the tumour area or altering M2 cell polarization by reprogramming to the M1 stimulatory phenotype.

1.2.1 Reducing M2 macrophages Recruitment and Survival

The first way to improve the immunosuppressive nature of the tumour area is to decrease the level of M2 macrophages (Table 1.1). Chemotherapy drugs have been reported to significantly reduce M2 cell survival, thereby reducing the

immunosuppressive activity within the TME, with the aim of limiting tumour growth (Table 1.1).

Doxorubicin (DOX) and its isomer Epirubicin (EPI) are widely used chemotherapy drugs in cancer treatment. They inhibit cell survival through intercalating DNA strands, thus inhibiting DNA/RNA synthesis, leading to cell death via triggering DNA cleavage by topoisomerase II. The level of M2 macrophages was reduced following delivery of DOX/EPI to M2 cells, resulting in tumour growth inhibition in breast cancer, colon carcinoma, and sarcoma (Zhu et al., 2022, Scialla et al., 2023, Sui et al., 2021, Wei et al., 2023).

Another anthraquinone chemotherapeutic agent, Mitoxantrone (MIT), has been shown to reduce M2 macrophages level. Nanoparticles (NPs) formulated using 2,2-bis(acryloyloxymethyl)propionate were designed for co-delivery of MIT and Celastrol (CEL) to kill M2 macrophages in melanoma-bearing animal models, and achieved significant inhibition of tumour growth (Liu et al., 2018).

Zoledronate (ZOL), a third-generation nitrogen-containing bisphosphonate, has shown selective cytotoxicity to TAMs (Tang et al., 2013). NPs containing ZOL achieved M2 macrophage cell death and sarcoma immunotherapy in tumour-bearing animal models (Zang et al., 2019). In addition, the co-delivery of EPI and ZOL has shown enhanced synergistic anti-tumour efficacy in sarcoma therapy (Sui et al., 2021).

Meanwhile, a photothermal agent, DiOC₁₈(7), formulated using a lipoprotein-based NPs significantly reduced the levels of both TAMs and TAFs within the tumour area following exposure to a 808 nm laser, which enhanced the second-wave treatment of the anti-cancer drug, mertansine, and limited breast cancer growth (Tan et al., 2019).

Table. 1.1: Chemotherapeutic strategies for depletion of M2 macrophages in cancer therapy.

Formulation	Drug	Cancer type & Animal Model	Ref.
Carnaúba wax lipid-based NPs	Doxorubicin (DOX)	S.C. MMTV-M-Wnt-1 breast cancer mouse	(Scialla et al., 2023)
Fe-doped NPs	DOX	S.C. CT26 colon cancer mouse	(Zhu et al., 2022)
Liposomes	Epirubicin (EPI)	S.C. S180 sarcoma mouse	(Wei et al., 2023)
Lipid-based NPs	Zoledronate (ZOL)	S.C. S180 sarcoma mouse	(Zang et al., 2019)
Liposomes	EPI and ZOL	S.C. S180 sarcoma mouse	(Sui et al., 2021)
2,2-	Mitoxantrone	Wnt5a ^{high} BRAF-	(Liu et al., 2018)

bis(acryloyloxymethyl)propionate NPs	(MIT) and Celastrol (CEL)	mutant melanoma mouse	
Lipoprotein-based NPs	DiOC18(7)	S.C. 4T1 breast cancer mouse	(Tan et al., 2019)

1.2.2 Promotion of TAM Polarization

In contrast to inducing M2 cell death, inducing the transformation of M2 cells into the M1 subtype is another immunotherapy strategy (Table 1.2). This approach has the advantage of not only reducing the level of immunosuppressive TAMs but also upregulating the expression of immunostimulatory factors and is thus more conducive to cancer immunotherapy.

The colony stimulating factor-1/colony stimulating factor-1 receptor (*CSF-1/CSF-1R*) signalling pathway has been identified as a key factor of TAMs polarization, and blockage of the *CSF-1/CSF-1R* signalling pathway can cause the reprogramming of M2 macrophages to the M1 phenotype. BLZ945, a *CSF-1R* inhibitor, has been reported to block *CSF-1R*, thus achieving reprogramming of M2 macrophages (Ramesh et al., 2019, Kulkarni et al., 2018, Ramesh et al., 2020). Furthermore, the application of RNA interference (RNAi) also presents a promising approach to decrease the expression of *CSF-1R*, when anti-*CSF-1R* small interference RNA (siRNA) was used to silence *CSF-1R* mRNA, the M2 macrophages were successfully re-educated (Qian et al., 2017, Li

et al., 2020). After reprogramming the TAMs, the TME is remodelled from an immunosuppressive environment to an immunostimulatory one, whereby M1 factors and the recruited cytotoxic T cells achieve tumour cell death. This novel immunotherapeutic strategy has been used in a variety of cancers including melanoma, breast cancer, and pancreatic cancer using tumour bearing mouse models (Table 1.2).

Bruton's tyrosine kinase (BTK) is overexpressed on M2 macrophages and has tumour-promoting effects (Weber et al., 2017). Ibrutinib (IBR), a BTK inhibitor, has been reported to block the expression of BTK, thus reprogramming TAMs, and limiting tumour growth in a sarcoma-bearing animal model (Qiu et al., 2019).

Signal-regulatory protein alpha (SIRP α) is overexpressed on TAMs and can interact with CD47, a transmembrane protein overexpressed on tumour cells, to release signals which negatively control phagocytosis by immune cells (Liu et al., 2019). Therefore, blockage of the CD47-SIRP α interaction has shown significant potential to reprogram TAMs and suppress tumour growth in animal models, Kulkarni et al., (2018) reported a novel strategy by combining a CSF-1R antibody and a SIRP α antibody. The dual-delivery of the two antibodies amplified the macrophage immune responses against aggressive cancer (breast cancer/melanoma) via blockage of both the CSF-1/CSF-1R pathway and the CD47-SIRP α interaction (Kulkarni et al., 2018).

In addition, other drugs have been shown to normalize TAMs polarization, such as

resiquimod (a TLR7/8 agonist) and the anti-programmed cell death protein 1 drugs (Rodell et al., 2018).

Table. 1.2: Strategies for reprogramming of M2 macrophages in cancer therapy.

Formulation	Therapeutic Agent	Cancer type & Animal Model	Ref.
Lipid-based NPs	Anti-CSF-1R siRNA	S.C. B16-F10 melanoma mouse	(Qian et al., 2017)
Nano micelles	Anti-CSF-1R siRNA and NVP-BEZ 235 (PI3K- γ inhibitor)	S.C. Pan 02 pancreatic cancer mouse	(Li et al., 2020)
Liposomes	Ibrutinib (a BTK inhibitor)	S.C. S180 sarcoma mouse	(Qiu et al., 2019)
Lipid-based supramolecular NPs	BLZ945 (a CSF-1R inhibitor) and selumetinib (a MEK inhibitor)	S.C. 4T1 breast cancer mouse	(Ramesh et al., 2020)
Lipid-based supramolecular NPs	BLZ945 and anti-SIRP α antibody	S.C. 4T1 breast cancer mouse/S.C. B16-F10 melanoma	(Kulkarni et al., 2018)

		mouse	
Lipid-based supramolecular NPs	BLZ945 and SHP099 (a SHP2 inhibitor)	S.C. 4T1 breast cancer mouse/S.C. B16-F10 melanoma mouse	(Ramesh et al., 2019)
Cyclodextrin-lysine polymeric NPs	Resiquimod (R848, an agonist of TLR7 and TLR8)	S.C. MC38 colon cancer mouse	(Rodell et al., 2018)
Cyclodextrin-lysine tri-monomer NPs	R848	S.C. MC38 colon cancer mouse; S.C. B16-F10 melanoma mouse	(Rodell et al., 2019)
Lipid-based NPs	Anti-CD47 and anti-SIRP α antibodies	S.C. 4T1 breast cancer mouse/S.C. B16-F10 melanoma mouse	Ramesh et al., 2020b

1.2.3 Targeting to M2 Macrophages

The most challenging part of the application of immunotherapy *in vivo* is the ability to achieve specific delivery to the M2 cells, thus specific M2 macrophage-targeting

ligands are urgently needed (Table 1.3). An artificially designed peptide, M2pep, when conjugated to nanoparticles based on polyethylenimine-stearic acid successfully achieved cell specific M2 macrophages targeting (Qian et al., 2017, Li et al., 2020). The M2pep sequence was selected by subtractive phage biopanning (Cieslewicz et al., 2013). In addition, an alternative M2 targeting ligand, α -peptide, which recognises the scavenger receptor B type 1 (SR-B1), that regulates phagocytosis and promotes the switch of M1 to M2 macrophages has been used in inflammatory immune responses (Zhang et al., 2019). NPs based on 1,2-dimyristoyl-sn-glycero3-phosphocholine and cholesterol oleate (DMPC/C.O) with an M2pep and an α -peptide bi-targeting ligands showed a higher M2 macrophages uptake than either of the ligands alone (Qian et al., 2017). The Siglec-1, a sialic acid (SA) receptor, overexpressed on M2 macrophages, is another ideal ligand. Drug delivery systems modified with SA enhanced cellular internalization in M2 cells via the SA-Siglec-1 axis (Li et al., 2021, Yan et al., 2022). The IL-4 receptor (IL4R) is overexpressed on M2 macrophages and is involved in M2 macrophage differentiation. The IL4R-binding peptide (IL4Rpep-1) when used as a targeting ligand achieved cell specific drug delivery to M2 macrophages (Gunassekaran et al., 2021). CD206 (also known as the mannose receptor) is an M2 macrophage marker has also been used as a receptor for ligands, it has a naturally high affinity with mannose. NPs based on PEGylated poly(lactic-co-glycolic acid) (PLGA) with mannose as the ligand containing a photosensitizer, indocyanine green, and an ROS photogeneration agent, titanium dioxide, successfully targeted and reprogramed M2 macrophages by phototherapy (Shi et al., 2018).

Table. 1.3: Ligands for specific targeting of M2 macrophages

Ligand	Receptor	Application	Ref.
M2pep	The receptor of M2pep is unclear	S.C. B16-F10 melanoma mouse/ S.C. Pan 02 pancreatic cancer mouse	(Qian et al., 2017, Li et al., 2020)
α -peptide	scavenger receptor B type 1 (SR-B1)	S.C. B16-F10 melanoma mouse	(Qian et al., 2017)
Sialic acid (SA)	Siglec-1 (CD169)	S.C. S180 sarcoma mouse	(Yan et al., 2022, Qiu et al., 2019, Ding et al., 2021, Sui et al., 2021)
Mannose	CD206	S.C. 4T1 breast cancer mouse/S.C. B16-F10 melanoma mouse	(Shi et al., 2018)
IL4Rpep-1	IL-4 receptor (IL4R)	S.C. 4T1 breast cancer mouse	(Gunassekaran et al., 2021)

In summary, immunotherapy is a novel and potentially effective treatment strategy for

cancer therapy, however it is still in the exploratory stage.

1.2.4 Other Immunotherapy Strategy

Different from the above immunotherapy targeting tumour-associated macrophages, another immunotherapy strategy is focusing on immune checkpoints. Inhibitory checkpoint molecules are targets for cancer immunotherapy due to their potential for use in multiple types of cancers. Currently, approved checkpoint inhibitors including block targeting programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1), and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) (Topalian et al., 2023). This approach is called immune checkpoint inhibitor (ICI) therapy. ICIs directly act on immune cells in the tumour microenvironment, inhibiting the expression of immune checkpoints, thereby activating the immune system and killing tumour cells (Riemann et al., 2020). However, this approach relies on the pre-existing activated cytotoxic T cells in the tumour microenvironment, once cancer has developed to the late stage, activated cytotoxic T cells have been depleted or destroyed, and the tumour microenvironment has become extremely immunosuppressive, its efficacy will rapidly decrease (Emens et al., 2017). Therefore, this approach only works for some specific cancer types.

1.3 Prostate Cancer

1.3.1 Prostate Cancer

Prostate cancer (PCa) is considered to be an immunosuppressive cancer and is the most

prevalent and second-deadliest cancer type in males. In US males approximately 268,490 new cases and about 34,500 deaths from prostate cancer were reported in 2022 which accounts for 27% of all new cancer cases and 11% of all cancer related deaths respectively (Siegel et al., 2022).

Mutations of genes involved in the androgen pathway and testosterone metabolism, are considered the main candidate genes associated with the development of PCa (Sekhoacha et al., 2022, Bluemn and Nelson, 2012). Thus, some of these specific genes have been used as biomarkers of PCa, which help with PCa diagnosis, prognosis, grading, evaluating of PCa metastasis and the choice of the therapeutic protocol. PCa mainly affects males aged between 45 and 60 years, and is diagnosed through prostate specific antigen (PSA) testing, prostate biopsy, and digital rectal examination (DRE) (Chen et al., 2013). DRE and PSA testing are the most common methods for PCa diagnosis. PSA is a glycoprotein, which exists in semen and blood and is released by prostate epithelial cells. Higher levels of PSA (> 10 ng/mL) in the blood suggested a higher PCa risk ($> 50\%$). It is reported that when the blood concentration of PSA is higher than 10 ng/mL, the probability of PCa diagnosis is more than half (Altwaijry et al., 2018). However, benign pathologies of the prostate also cause higher PSA levels consequently a prostate biopsy is required to confirm a PCa diagnosis. Currently, several novel imaging systems have been developed for diagnosis of PCa, including prostate-specific membrane antigen positron emission tomography scans, which improve the accuracy and timeliness of PCa diagnosis (Kasivisvanathan et al., 2018).

1.3.2 Prostate Cancer Grading

TNM system is a common staging description tool for PCa. The system was developed by the American Joint Committee on Cancer and uses T(primary tumour), N (regional lymph nodes), and M (distant metastasis) to label the progress of PCa. The T refers to the size and extent of the primary tumour; the N refers to the number of nearby lymph nodes which have cancer; and the M refers to whether the PCa has metastasized (www.cancer.gov).

Except for the TNM staging system, PCa is also graded by a Gleason score. The Gleason score was created by Donald F. Gleason in 1966 based solely on the architectural pattern of the tumour (Gleason and Mellinger, 1974). The innovative aspect of this system was, rather than assigning the worst grade as being the grade of the carcinoma, the grade was defined as the sum of the two most common grade patterns and reported as the Gleason score (Epstein, 2016). The Gleason scoring system is the most widely used grading system for PCa. 2 different areas of the prostate will be observed under a microscope and a scale of 3 – 5 will be given to describe the pathomorphology of the cancer cells. The greater the difference between tumour cells and healthy cells, the higher the Gleason score is given. In other words, the tumour cells exhibit more aggressive, the higher the Gleason score receives in the area. The scores of 2 areas are added to come up with an overall Gleason score, which is between 6 and 10 (www.cancer.net).

PCa is generally grouped into 4 stages, Stage I to IV, based on the TNM system, the Gleason score, and PSA levels:

Stage I: the PCa is slow-growing, the tumour is unable to be felt and involves 50% of either side of the prostate or less. PSA levels are low, and the tumour cells are similar to the healthy prostate cells on morphocytology

Stage II: the tumour is only detected in the prostate, with medium or low PSA levels. The tumour in this stage is small but was thought to have an increasing risk of growing.

Stage II can be subdivided into 3 sub-stages:

Stage IIA: The tumour is unable to be felt and involves 50% of either side of the prostate or less, with medium PSA levels. The Gleason score indicates the tumour cells are well differentiated. Or the tumour is only detected in the prostate, with the tumour cells being well differentiated.

Stage IIB: The tumour is only detected inside the prostate, and the tumour is large enough to be felt during a DRE. The PSA level is medium. The tumour cells are moderately differentiated.

Stage IIC: The tumour is only detected inside the prostate, and the tumour is large enough to be felt during a DRE. The PSA level is medium. The cancer cells may be moderately or poorly differentiated.

Stage III: PSA levels are high, the tumour is growing, or the Gleason score is high. These all indicate a locally advanced cancer that is likely to grow and metastasise.

Similar to Stage II, Stage III is subdivided into 3 sub-stages based on various situations.

Stage IIIA: PCa has metastasised beyond the outer layer of the prostate into nearby tissues, and has possibly metastasised to the seminal vesicles. The PSA level is high.

Stage IIIB: The tumour has metastasised outside of the prostate gland and has possibly invaded nearby structures, such as the bladder or rectum.

Stage IIIC: The Gleason score of the cancer is high, which means that the tumour cells are poorly differentiated.

Stage IV: The cancer has metastasised beyond the prostate. This is the late stage in this grading system and can be subdivided into two sub-stages depending on the situation.

Stage IVA: The cancer has metastasised to the regional lymph nodes.

Stage IVB: The cancer has metastasised to distant lymph nodes, other parts of the body, or to the bones.

In 2016, a new PCa grading system was recommended by the editor of five major urology journals, the system categorizes tumours from Grade group 2 (least aggressive) to Grade group 5 (most aggressive) (Epstein, 2016). This new grading system is based on the Gleason score and uses 5 grade groups to accurately reflect the prognosis for both the needle biopsy and radical prostatectomy: Grade group 1: Gleason score 2 – 6; Grade group 2: Gleason score 3 + 4 = 7; Grade group 3: Gleason score 4 + 3 = 7; Grade group 4: Gleason score 8; and Grade group 5: Gleason score 9 – 10 (Table 1.4) (Pierorazio et al., 2013).

Table 1.4: Histological definition of the new grading system (Epstein et al., 2016)

Grade Group	Gleason Score	Description
1	≤ 6	Only individual discrete well-formed glands.
2	$3 + 4 = 7$	Predominantly well-formed glands with lesser components of poorly formed/fused/cribriform glands.
3	$4 + 3 = 7$	Predominantly poorly formed/fused/cribriform glands with lesser components of well-formed glands.*
4	$4 + 4 = 8$ $3 + 5 = 8$ $5 + 3 = 8$	Only poorly formed/fused/cribriform glands. [‡] Or predominantly well-formed glands and lesser components lacking glands. [‡] Or predominantly lacking glands and lesser components of well-formed glands.
5	9 – 10	Lacks gland formation (or with necrosis) with or without poorly formed/fused/cribriform glands*

*For cases with >95% poorly formed/fused/cribriform glands or lack of glands on a core or at radical prostatectomy, the component of <5% well-formed glands is not factored into the grade.

[‡]Poorly formed/fused/cribriform glands can be a more minor component.

1.3.3 Stage-specific Standard Care for Prostate Cancer

Different treatments are recommended for each stage of PCa.

Early-stage PCa (including Stages I and II): Early-stage PCa normally grows slowly and possibly takes years to cause symptoms. Therefore, active surveillance, watchful waiting, radiation therapy (external beam or brachytherapy), surgery, and treatment in clinical trials are recommended. American Society of Clinical Oncology (ASCO), the American Urological Association, and the Society of Urologic Oncology recommend that patients with high-risk early-stage PCa that has not spread to other areas should receive radical prostatectomy or radiation therapy with Androgen-deprivation therapy (ADT) as standard treatment options.

Locally advanced PCa (Stage III): Patients in Stage III PCa can be treated with external-beam radiation therapy and concurrent ADT or surgery. ASCO recommends that patients who receive ADT combine abiraterone and prednisolone to stop testosterone production. Once abiraterone is not applicable, combined androgen blockade using AR inhibitors is recommended. For patients who have received surgery, postoperative adjuvant (salvage) radiation therapy is a standard of care for PCa with extraprostatic extension.

Advanced PCa (Stage IV): Generally, metastatic PCa is regarded as advanced PCa.

Locally advanced PCa (Stage III) has a great chance of developing into metastatic PCa.

For advanced PCa, clinical trials may be an option. To date, there is no cure for metastatic PCa, therefore, new therapeutic strategies are urgently needed for advanced PCa. Immunotherapy, especially tumour-associated macrophage-targeted immunotherapy may become a potential treatment for advanced prostate cancer.

1.4 RNA Interference (RNAi) Mechanism

1.4.1 RNAi and siRNA

RNAi is a gene silencing process using double stranded RNA (dsRNA) and was first discovered by Richard Jorgensen in 1990. RNAi is an evolutionarily conserved mechanism which plays an innate immune defensive role in protecting against harmful effects of invasive nucleic acids from viruses, transposable elements and other pathogens (Guo et al., 2010). Small RNAs, such as microRNA (miRNA) and small interference RNA (siRNA), promote RNAi pathways. miRNA induces translational suppression and transcript deprivation for imperfectly complementary messenger RNA (mRNA), whereas siRNA mediates gene knockdown via sequence-specific cleavage of perfectly matching mRNA targets (Dorsett and Tuschl, 2004). This approach can selectively silence proto-oncogenes causing apoptosis of cancer cells with the aim of treating the disease. Downregulating the expression of oncogenes has been confirmed to limit PCa growth and metastasis (Lu, 2009, Mimeault and Batra, 2011). Figure 1.2 summarises three mechanisms to knockdown endogenous mRNA using the RNAi pathway (Guo et al., 2010) .

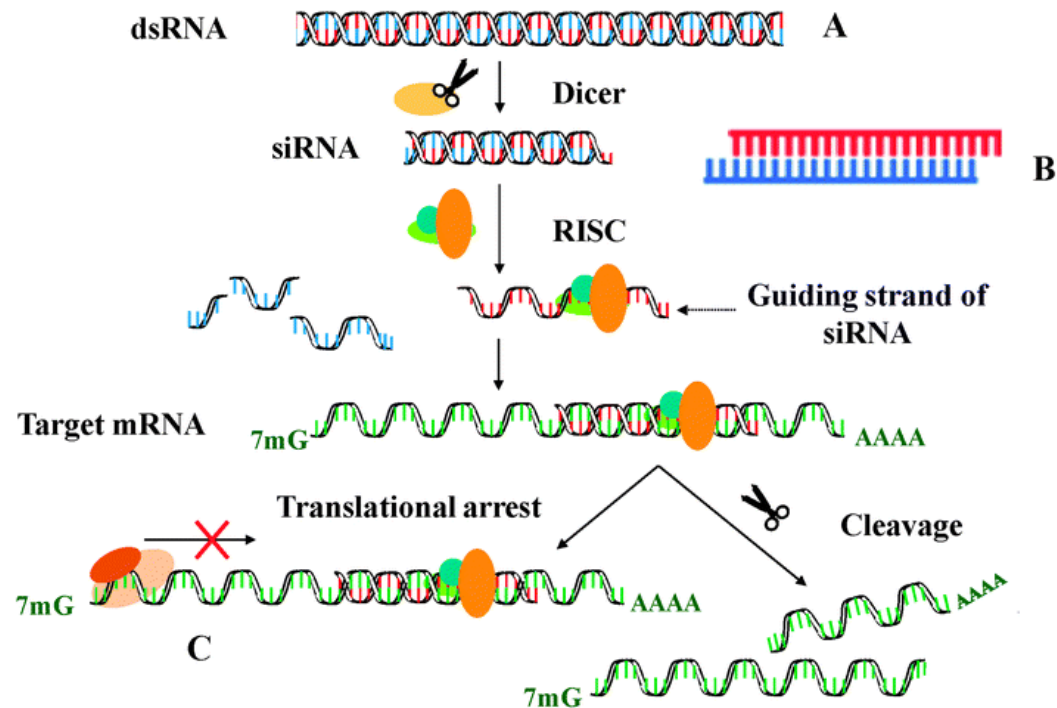


Figure 1.2 Knockdown of endogenous mRNA using the RNAi pathway. (A) longer dsRNAs can be transfected into the cytoplasm, follow Dicer processing they are recognized by RNA-induced silencing complex (RISC) define, (B) synthetic siRNAs can be transfected into cells and loaded into RISC directly for the RNAi pathway, and (C) miRNA blocks translation with a less perfectly matched sequence (Guo et al., 2010).

1.4.2 Challenges for the Therapeutic Application of siRNA

The challenges to translating the concept of gene silencing using siRNA into the clinic include; off-targeted effects, stability, and above all the ability to deliver siRNA to the right cells, at the right time, at the correct concentration.

The off-targeted effects of siRNA include a) non-specific silencing, and b) initiation or enhancement of the innate immune system. Methods to avoid off-targeted effects when

using siRNA include: a) avoiding the use of immuno-stimulatory sequence motifs and high uridine content (Hajeri and Singh, 2009), b) nullification of the immune response by introducing modifications in the nucleotides and/or their backbone while maintaining silencing activity (Sioud, 2006, Judge et al., 2006), and c) ensuring that siRNA has a 2-nt 3' overhang, as siRNAs without such overhangs activate the interferon system (Marques et al., 2006).

Secondly, the stability of siRNA is another challenge, as serum nucleases, which lead to siRNA degradation, are widely present in serum and plasma. Chemical modification of siRNA can improve its stability, for example conjugation of a phosphorothioate backbone at the 3' end and 2' modifications can protect against degradation (Soutschek et al., 2004).

The next and most significant challenge is the delivery of siRNA to the diseased cells. The delivery of siRNA faces two significant obstacles, at the extracellular and intracellular levels. Due to the physicochemical properties of siRNA including a high molecular weight, the hydrophilic nature and the negative charge it is unable to enter the cell through the lipid bilayer of the membrane, consequently a delivery system is necessary (Bumcrot et al., 2006). Non-viral delivery of RNA has been explored and recent successes reaching the market include Patisiran (Onpattro™) (siRNA) for liver disease administered by IV injection and the Covid 19 mRNA vaccine, administered IM, both formulated in lipid-based nanoparticles (Akinc et al., 2019). In addition, the

nanoparticle non-viral approach provides the opportunity to incorporate targeting ligands for cell specific delivery (Guo et al., 2017).

1.5 RNA-based therapeutics for the treatment of PCa

Recently, siRNA-based gene therapy has been applied to the treatment of PCa with some promising results (Table 1.5). Several genes associated with PCa formation have been identified, such as RPL19 (ribosomal protein L19e), Arrestin2, and transcription intermediary factor 2 (TIF2) (Agoulnik et al., 2006, Lakshmikanthan et al., 2009, Bee et al., 2011). It has been shown that biomimetic tumour-targeting magnetic lipid NPs containing dihomono- γ -linolenic acid (DGLA) and 2,4-dienoyl-CoA reductase 1 (DECR1) siRNA significantly reduced PCa growth in a nude mouse model with no distant organ metastasis (Chen et al., 2022). The androgen receptor (AR) plays an important role in advanced-stage PCa. Quick et al. reported that lipid NPs containing AR siRNA significantly inhibited PCa growth and prolonged survival in 22Rv1 PCa-bearing mouse models (Quick et al., 2022). In addition, it has been shown that S-phase kinase-associated protein 2 (Skp2) siRNA and quercetin (Que) self-assembled NPs could accumulate in the PCa area and limit tumour growth in a nude PCa mouse model (Liang et al., 2022). Angiogenesis plays an important role in the development of PCa, thus specific downregulation of angiogenesis-related genes expression could limit PCa growth. Delivery of vascular endothelial growth factor (VEGF) siRNA using a cyclodextrin-based NP to a PCa-bearing animal model reduced VEGF mRNA expression and reduced PCa growth (Guo et al., 2012). In addition, placental growth

factor (PlGF), which is a member of the VEGF family and is released from carcinoma-associated fibroblasts, is overexpressed following androgen deprivation therapy in PCa. Delivery of PlGF siRNA downregulated the expression of PlGF and suppressed PCa growth in a mouse model (Zins et al., 2013). Recent research also revealed the relationship between interleukin-8 (IL-8) and angiogenesis in PCa. Systemic and local intra-tumour administration of IL-8 siRNA significantly inhibited PCa growth and tumour sections revealed the neo-vasculature was destroyed post-administration of IL-8 siRNA (Aalinkeel et al., 2016). Similarly, the inhibition of HIF-1- α /Notch-1 signaling pathway via downregulation of eIF-2- α or GHET1 by siRNA affected angiogenesis, and subsequently limited PCa growth (Zhu et al., 2019).

Table 1.5: siRNA-based therapeutics for PCa treatment.

Formulation	siRNA	PCa model	Ref
biomimetic tumor-targeting magnetic lipid NPs	DGLA/DECR1 (i.v.)	PCa-bearing nude mouse with high- fat diet	(Chen et al., 2022)
Luteinizing hormone- releasing hormone(LHRH) analog-tethered	MCL-1 (i.v.)	LNCaP xenografted mouse	(Tambe et al., 2023)

multi-layered polyamidoamine (PAMAM) nanoconstructs			
Lipid NPs	Androgen receptor (AR) (i.v.)	22Rv1 PCa- bearing mouse	(Quick et al., 2022)
Supramolecular NPs	Skp2 (i.v.)	PCa-bearing nude mouse	(Liang et al., 2022)
/	PlGF (i.tu.)	S.C. PCa mouse	(Zins et al., 2013)
Biocompatible, cationic polylactide nanocarriers	IL-8 (i.v.)	S.C. PCa mouse	(Aalinkeel et al., 2016)
Cationic lipid/neutral lipid bilayer and hydrophilic polymers	KLF5 (i.v.)	S.C. PCa mouse	(Yagi et al., 2009)
Gold NPS capped with polyethylenimine	RelA (i.p.)	S.C. PCa mouse	(Luan et al., 2019)

(PEI) and PEGylated anisamide			
pH-responsive polymer	BRD4 (i.v.)	S.C. LNCaP xenograft PCa model	(Xu et al., 2017)
Lipofectamine 2000 transfection agent	GHET1	LNCap/C4-2 cells	(Zhu et al., 2019)
Cyclodextrin-based NPs			
β -cyclodextrin	PLK1	PC3/DU145 cells	(Malhotra et al., 2018)
Folate-targeted amphiphilic cyclodextrin	ZEB1/NRP1	LNCaP/PC3 3D PCa model	(Evans et al., 2017b)
Anisamide- targeted amphiphilic cyclodextrin	PLK1	PC3/DU145 3D PCa bone metastases model	(Evans et al., 2017a)
Cationic cyclodextrin	RelA	PC3/LNCaP co- cultures, in 2D/3D PCa	(Fitzgerald et al., 2016a)

		model	
Folate-targeted amphiphilic cyclodextrin	RelA	VCaP/LNCaP cells	(Evans et al., 2016)
Hepta-guanidino- β -cyclodextrin	VEGF (i.v.)	PCa xenografted mouse	(Guo et al., 2012)

i.v.: intravenous injection; i.p.: intraperitoneal injection; i.tu.: intratumoral injection.

In addition to siRNA, microRNAs (miRNAs) are also capable of gene silencing. MiRNAs are small non-encoded single-stranded molecules, which could regulate the expression of a particular gene at the transcription stage. The regulatory patterns of miRNA is more complex than siRNAs, which makes them suitable for fine-tuning gene expression in different tissues (Table 1.6) (Sidorova et al., 2023). Some specific miRNAs have been identified as possible therapeutic targets for PCa treatment, including let-7 family, especially miRNA Let-7c (Mulholland et al., 2019) and miRNA 34a (Chalanqui et al., 2016).

Table 1.6: The differences between miRNA and siRNA (Sidorova et al., 2023).

	miRNA	siRNA
Structure	small (18–25 nucleotides) non-coding single-stranded molecules	small (21–23 nucleotides) non-coding double-stranded molecules
Target	Many DNA/mRNA molecules	One mRNA molecule

Sites	Cytoplasm, nucleus	Cytoplasm
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1.6 Cyclodextrin for Gene Delivery

As mentioned above, the main barrier to RNAi therapy is delivering the negatively charged and hydrophilic RNA molecules into targeted cells. Viral vectors have been widely used in gene delivery (Whitehead et al., 2009, Scholz and Wagner, 2012). However, for reasons of safety, mutagenesis, oncogenesis, and commercial production, the development of non-viral vectors as an alternative strategy is urgently needed and is gaining traction (Schaffert and Wagner, 2008, Li and Huang, 2006). While to date materials based on cationic lipids and polymers have been the most widely studied non-viral approaches for therapeutic nucleic acid delivery, modified cyclodextrins have also shown promise (Haley et al., 2020, O'Mahony et al., 2013).

Cyclodextrins (CDs) are a family of cyclic oligosaccharides derived from starch, with hydrophilic primary and secondary faces and a hydrophobic cavity (Fig. 1.3b). CDs have been well well-characterised as pharmaceutical excipients (Davis and Brewster, 2004). According to the number of glucopyranose units, CDs are classified to α -, β -, and γ -CD, where individual glucopyranose units are linked by α -1,4-glycosidic linkages (Loftsson et al., 2005, Ortiz Mellet et al., 2010) (Fig. 1.3a).

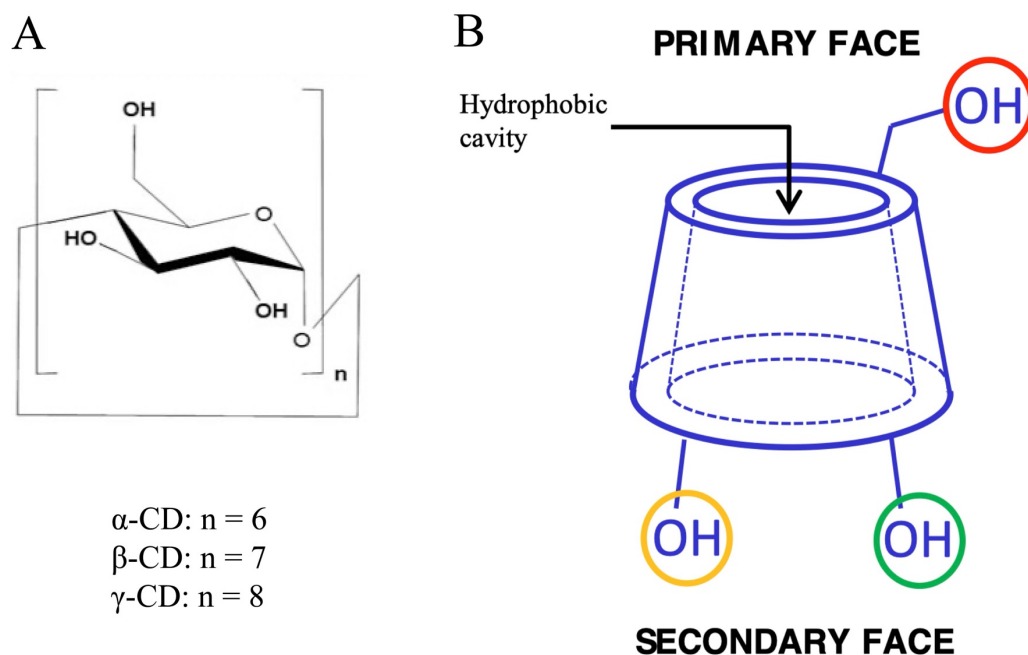


Fig. 1.3 (a) Cyclodextrin chemical structure, where 'n' represents the number of glucopyranose units in the ring, (b) Schematic of the CD structure, depicting the hydrophobic cavity for inclusion of guest molecules and the primary and secondary hydroxyl groups which have potential for chemical modification (O'Mahony et al., 2013).

CDs have traditionally been used to improve the solubility and subsequent bioavailability of low molecular weight hydrophobic drugs via inclusion complex formation (Uekama et al., 1998). However more recently, the application of CDs as non-viral delivery vectors for hydrophilic nucleic acids has been explored delivery (Chaturvedi et al., 2011, Ortiz Mellet et al., 2011).

Calando Pharmaceuticals were the first to advance a cyclodextrin containing siRNA/Oligonucleotide Nanoparticle Delivery (RONDEL™) technology for clinical application. RONDEL™ was based on linear cyclodextrin-containing polymers

(CDPs), first developed in 1990s. CALAA-01, the first candidate, containing M2 subunit of ribonucleotide reductase (RRM2) siRNA was submitted to the Food and Drug Administration (FDA) for a phase I trial in solid tumour patients, and approved in 2008. This was the first RNAi application in humans (Heidel and Schluep, 2012). The CDs were used as part of the delivery systems, to help reduce the toxicity and to facilitate PEGylation of the system via adamantane-PEG inclusion complex formation (Davis, 2009).

A further advantage of the CD structure is the presence of hydroxyl groups on the ring structure which can be modified resulting in functionalised cyclodextrins designed specifically for different delivery requirements including nucleic acid delivery (Ortiz Mellet et al., 2011). In previous studies in our laboratory, a series of modified CDs including cationic amphiphilic (O'Mahony et al., 2012b), anionic (Kont et al., 2022), neutral/PEGylated (Godinho et al., 2014), and targeted (Gooding et al., 2015) derivatives have been developed and their biocompatibility and transfection efficiencies have been evaluated in a range of cell lines and diseased animal models (Figure 1.4 and Table 1.4) (Guo et al., 2010, Guo et al., 2012, Godinho et al., 2013, McCarthy et al., 2013, O'Mahony et al., 2013, Fitzgerald et al., 2015, Evans et al., 2016, Evans et al., 2017a, Evans et al., 2017b, Guo et al., 2017, Malhotra et al., 2018, Mendonca et al., 2021, Zou et al., 2021, Kont et al., 2022).

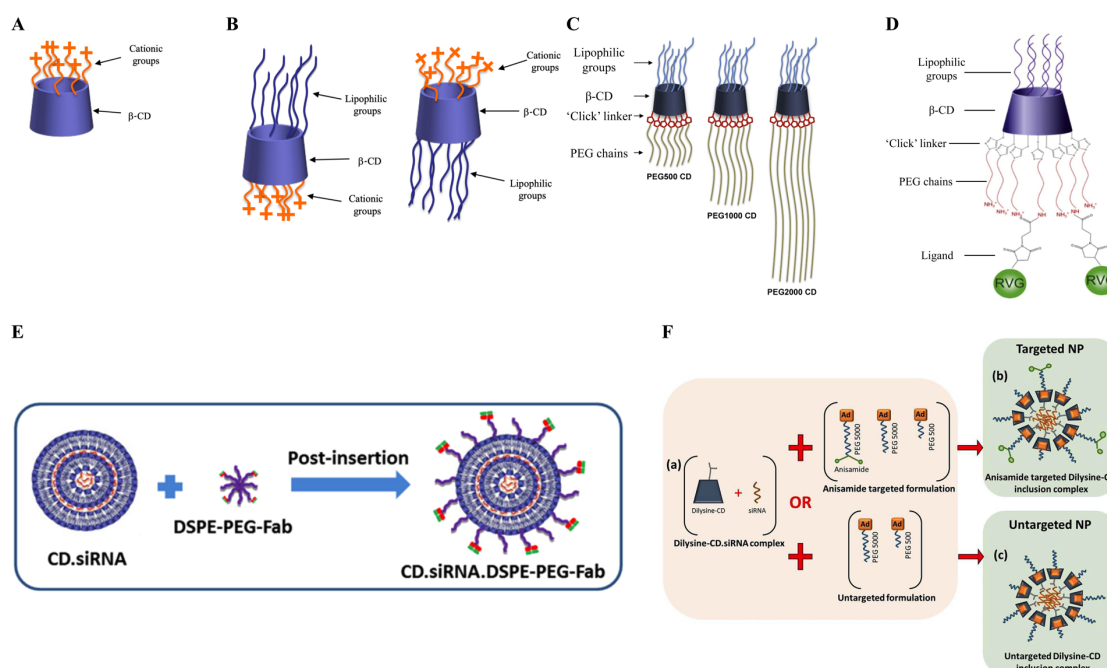


Fig. 1.4. Functionalised CD gene as siRNA delivery vectors. (A) Cationic CD, (B) amphiphilic cationic and inverted amphiphilic cationic CD, (C) neutral PEGylated amphiphilic CD, (D) Rabies Virus Glycoprotein (RVG)-CD covalently linked ligand, (E) non-covalent Fab targeted CD, and (F) exploitation of β -CD inclusion complex with adamantane-PEG-ligand.

In the earliest study, the most effective cationic β -CD identified was the pyridyl-amino (Fig. 1.4a) (Cryan et al., 2004). Subsequently, amphiphilic cationic CDs based on this cationic CD were developed, where the hydroxyl group on the primary face of the cationic CD was modified with an aliphatic chain, while the secondary hydroxyl group was modified by a short oligoethylene glycol amine (Fig. 1.4B). In addition, an “inverted” amphiphilic β -CD with the lipophilic chains on the secondary side was reported (Fig. 1.4B) (Byrne et al., 2009, MJ et al., 2011, Godinho et al., 2013). This “inverted” amphiphilic β -CD led to higher uptake and greater gene silencing in a

hypothalamic neuronal cell line, which may be due to changes in self-assembly properties (O'Mahony et al., 2012a). Based on the amphiphilic cationic β -CD, a PEGylated β -CD was developed to increase stability in salt-containing media and reduce surface charges in order to decrease cytotoxicity (Fig. 1.4C) (Godinho et al., 2014). The stability of nanoparticles co-formulated with the PEGylated β -CD increased with the length and density of PEG chains.

In addition, due to the flexibility of the CD ring structure it is possible to construct targeted β -CDs as the non-viral vectors for siRNA to enhance the efficacy in specific diseases conditions. Gooding et al. described a rabies virus glycoprotein (RVG)-modified CD designed to target the Central Nervous System (CNS) for use in glioblastoma treatment (Fig. 1.4D) (Gooding et al., 2015). This RVG-targeted ligand was covalently linked to the CD. Results showed that the targeted CD enhanced the cellular uptake compared with non-targeted formulations and achieved impressive gene knockdown in human glioblastoma cells.

The incorporation of a targeting ligand to CDs by non-covalent means is also possible using a 'post-insertion' method (Fig. 1.4E). This formulations approach involves two steps, firstly, siRNA is incubated with for example an amphiphilic cationic CDs to form non-targeted CD NPs, subsequently the ligand is incorporated by co-formulation with a targeted PEGylated lipid such as DSPE-PEG. Post-insertion has been widely used to modify the surface of liposomes (Iden and Allen, 2001), which allowed PEG chains to

transfer into the liposomal or lipidic bilayer (Perrier et al., 2010). These targeted CDs have been reported the successful use in the treatment of a series of diseases, including acute myeloid leukemia (AML), and Huntington's disease (HD). Guo et al. described an IL-3R α antibody-targeted CD-based formulation containing BRD4 siRNA to target IL-3R α , which is overexpressed on the surface of AML cells. Results showed this formulation could specifically transfer BRD4 siRNA to AML cells, including KG1 cell line and primary samples (patients blood), downregulating BRD4 expression, and causing apoptosis of AML cells (Guo et al., 2017). Mendonça et al. developed a RVG-targeted CD-based NP containing human huntingtin-targeting (mHTT antisense oligonucleotides (ASOs), which was achieved by post-insertion. The targeted CD downregulated mHTT expression and was capable of permeating an *in vitro* model of the blood-brain barrier (BBB) (Mendonça et al., 2023).

A third method to construct targeted cyclodextrins is by using an adamantane-PEG-targeting ligand construct which forms an inclusion complex in the hydrophobic cavities of the cyclodextrin (Fig. 1.4E). Fitzgerald et al. described an anisamide-targeted CD vector aimed at the sigma-1 receptor overexpressed on the surface of prostate cancer cells for the treatment of prostate cancer (Fitzgerald et al., 2016b). The targeted formulation mediated higher cellular uptake in three different PCa cell lines including PC-3, DU145, and VCaP, downregulating PLK1 expression, and promoting cell apoptosis. In addition, this targeting approach successfully delivered, across an *in vitro* BBB model, mHTT siRNA for the treatment of HD where adamantane-RVG was

the targeting ligand (Mendonca et al., 2021)

A range of other modified CD have been used to deliver therapeutic nucleic acid as summarised in Table 1.7.

Table 1.7: Applications of cyclodextrin for therapeutic nucleic acid delivery.

Modified CD	Therapeutic nucleic acid	Disease type	Ref.
Transferrin targeted and PEGylated CD	EWS FLI1 siRNA	Sarcoma	(Hu-Lieskovan et al., 2005)
Transferrin targeted and PEGylated CD	RRM2 siRNA	Neuroblastoma	(Bartlett and Davis, 2008)
lactosylated dendrimer/ α -CD	transthyretin siRNA	Familial amyloidotic polyneuropathy	(Hayashi et al., 2012)
β -CD coupled to amino acids	MDR1 siRNA	Cervical cancer	(Li et al., 2012)
‘Click’ modified amphiphilic and cationic β -CD	Htt siRNA	Huntington's disease	(Godinho et al., 2013)

Amphiphilic cationic β -CD	TNF- α siRNA	Acute colitis	(McCarthy et al., 2013)
Low molecular weight polyethyleneimine cross-linked with 2-hydroxypropyl- β -CD	VEGF siRNA	Cervical cancer	(Li et al., 2013)
Mesoporous silica nanoparticle functionalized with CD-grafted polyethylenimine	PKM2 siRNA	Adenocarcinoma	(Shen et al., 2014)
CD derivative consisting of a β -CD core and poly(l-lysine) dendron arms	MMP-9 siRNA	Nasopharyngeal cancer	(Liu et al., 2014)
Cationic star-shaped polymer consisting of β -	MMP-9 siRNA	Wound healing	(Li et al., 2014)

CD core and poly(amidoamine) dendron arms			
6-O- α -(4-O- α -D-glucuronyl)-D-glucosyl- β -CD conjugate with polyamidoamine dendrimer	TTR siRNA	Familial amyloidotic polyneuropathy	(Anno et al., 2014)
Neutral PEGylated amphiphilic CD	HTT siRNA	/	(Godinho et al., 2014)
RVG-CD covalently linked ligand	GAPDH siRNA	Human glioblastoma	(Gooding et al., 2015)
CD-grafted polyethylenimine	p65 siRNA	Breast cancer	(Wang et al., 2016)
Folate-modified star-shaped copolymer consisting of β -CD and poly(L-	MMP-9	Nasopharyngeal cancer	(Liu et al., 2016)

lysine) dendron			
Antibody-targeted β -CD	BRD4 siRNA	Acute myeloid leukemia	(Guo et al., 2017)
Polyethyleneimine e-modified (2-hydroxypropyl)- γ -CD	ETS1 siRNA	Multidrug-resistant breast cancer	(Wu et al., 2017)
β -CD-modified dendrimer-entrapped gold nanoparticles	Bcl-2/VEGF siRNA	Glioblastoma	(Qiu et al., 2018)
Molecular multivalent amphiphilic β -CD	CLTC/CAV1/PAK1 siRNA	Glioblastoma	(Manzanares et al., 2020)
CD-based biological porous nanospheres	EpCAM siRNA	hepatocellular carcinoma	(Chen et al., 2020)
Rabies virus glycoprotein-targeted β -CD	Htt siRNA	Huntington's disease	(Mendonca et al., 2021)
Folate-targeted PEGylated CD	RelA siRNA	Colorectal cancer	(Zou et al., 2021)

Delphinidin-modified β -CD	HMOX1 siRNA	Inflammatory pain	(Sauer et al., 2021)
Amphiphilic Cationic and Anionic β -CD	BRD4 siRNA	Acute myeloid leukemia	(Kont et al., 2022)
Polycationic amphiphilic β -CD derivative	P42-MAPK/Rheb siRNA	Prostate cancer	(de la Torre et al., 2022)
Redox-sensitive folate-appended-polyethylenimine- β -CD	hTERT siRNA	Cancer	(Mousazadeh et al., 2022)
RVG-targeted β -/ γ -CD	HTT ASOs	Huntington's disease	(Mendonca et al., 2023)

In addition to the cationic CDs mentioned above, cationic CD polymers are also used in gene delivery and have been shown to electrostatically interact with nucleic acids to form stable nano-complexes (Pun et al., 2004, Ortega-Caballero et al., 2008, Mendez-Ardoy et al., 2011, Ortiz Mellet et al., 2011). Polymers have been designed with long shelf lives, which may be suitable for clinical applications (Olden et al., 2018). It is reported that cationic CD polymers utilise the proton sponge effect to achieve endosomal escape (Haley et al., 2020, Wojnilowicz et al., 2019).

In summary, CDs are widely used in the food, cosmetics, and pharmaceutical industries due to their low toxicity and low immunogenicity. Modified CDs have been utilised as non-viral nucleic acid delivery vectors. In particular, amphiphilic cationic CDs have been shown to successfully deliver therapeutic nucleic acids to a variety of cell types including cancer, and neuronal cells with proof of concept established in diseased animal models of prostate cancer, IBD and Huntington's disease. The results to date indicate the potential for clinical translation of CD-based delivery systems.

However, CD-based nanoparticles are still in the early testing phases and require further investigations, several clinical trials on CD-based nanoparticles in the past 20 years have reported promising results for cancer therapy, including: clinical trial Nos.: NCT01612546 (Phase 2, completed, using cyclodextrin-based polymer-camptothecin CRLX101 for advanced or metastatic stomach, gastroesophageal, or esophageal cancer which cannot be removed by surgery) and NCT01803269 (Phase 2, completed, using cyclodextrin-based polymer-camptothecin CRLX101 for recurrent small cell lung cancer).

1.7 Objective and Aims of the Thesis

1.7.1 Overall objective:

To develop and compare a range of amphiphilic cationic β -cyclodextrin-based nanoparticulate siRNAs delivery systems to specifically target M2 macrophages by

sialic acid and M2pep, to reprogram the immunosuppressive tumour-associated macrophages, M2 cells to the immunostimulatory M1 phenotype by silencing the expression of CSF-1R mRNA via RNA interference. The reprogrammed TAMs secreted M1 factors including chemokines and cytokines to cause PCa cells apoptosis and recruit cytotoxic T cells, thereby remodelling the immunosuppressive nature of the TME, with the ultimate purpose of achieving improved treatment of prostate cancer by exploiting immunotherapy.

1.7.2 Aim 1:

To synthesise the sialic acid-based targeting ligand and conjugate it with cationic amphiphilic cyclodextrin via a post-insertion method. To evaluate the targeting and tumour inhibitory effects of the resulting NPs containing CSF-1R siRNA in cell culture models of both human and mouse macrophages and PCa cells (Chapter 2).

1.7.3 Aim 2:

To covalently conjugate the M2pep targeting ligand to DSPE-PEG-Maleimide using the Michael reaction. To incorporate the M2pep targeting construct into a cationic amphiphilic cyclodextrin by the post-insertion method. To evaluate the M2 macrophage-targeting and TAMs reprogramming effects of the resulting NPs with CSF-1R siRNA in both human and mouse macrophages, and to quantify the anti-prostate cancer effects in human and mouse PCa cells *in vitro* (Chapter 3).

1.7.4 Aim 3:

To compare the efficacy of the targeted NPs produced, to identify the optimum prototype and evaluate the efficacy and mechanism of action *in vivo* using a PCa-bearing mouse. To monitor the biodistribution of the NPs, to estimate the *in vivo* toxicity, and to quantify the TME remodelling and the TAMs reprogramming effects (Chapter 4).

1.8 References

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Chapter 2 Sialic acid-targeted cyclodextrin-based nanoparticles deliver CSF-1R siRNA and reprogram tumour-associated macrophages for immunotherapy of prostate cancer

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2.1 Abstract

Prostate cancer remains a serious condition threatening the health of men. Due to the complicated nature of the tumour microenvironment (TME), conventional treatments face challenges including poor prognosis and tumour resistance, therefore new therapeutic strategies are urgently needed. Small interfering RNA (siRNA), a double-stranded non-coding RNA, regulates specific gene expression through RNA interference. Tumour-associated macrophages (TAMs) are a potential therapeutic target in cancer immunotherapy. Colony stimulating factor-1/colony stimulating factor-1 receptor (CSF-1/CSF-1R) signaling pathway plays a crucial role in the polarization of the immunosuppressive TAMs, M2 macrophages. Downregulation of CSF-1R is known to reprogram the immunosuppressive TAMs, M2 macrophages, to the immunostimulatory phenotype, M1 macrophages. Sialic acid is a ligand for Siglec-1 (CD169) which is overexpressed on M2 macrophages with little expression in other phenotypes. Therefore, a sialic acid-targeted cyclodextrin-based nanoparticle was developed to specifically deliver CSF-1R siRNA to M2 macrophages. The nanoparticles were studied *in vitro* using both human and mouse prostate cancer cell lines. Results show that the targeted nanoparticles achieved cell specific delivery to M2 macrophages via the sialic acid-CD169 axis. The expression of CSF-1R was significantly downregulated in M2 macrophages (29.64% for targeted vs 19.31% for non-targeted nanoparticles in THP-1-derived M2 macrophages and 38.94% for targeted vs 18.51% for non-targeted nanoparticles in RAW 264.7-derived M2 macrophages, $n = 4, p < 0.01$). The resulting reprogramming of M2 macrophages to M1 enhanced the level

of apoptosis in the prostate cancer cells in a Transwell model (49.17% for targeted vs 37.68% for non-targeted nanoparticles in PC-3 cells and 69.15% for targeted vs 44.73% for non-targeted nanoparticles in TRAMP C1 cells, $n = 3$, $p < 0.01$). Thus, this targeted cyclodextrin-based siRNA drug delivery system provides a potential strategy for prostate cancer immunotherapy.

2.2 Keywords

Modified cyclodextrins; RNA interference; sialic acid; tumor-associated macrophages; prostate cancer; immunotherapy.

2.3 Introduction

Prostate cancer (PCa) is the most common cancer in western men. In 2022, approximately 27% of new cancer diagnosis and 11% of cancer related deaths in American males were caused by PCa, which has the highest incidence and the second-highest death rate after lung cancer (Siegel et al., 2022). If diagnosed at an early state, PCa can be treated with a 5-year survival rate of about 95%, but once PCa develops to the advanced stage, the 5-year survival rate decreases to less than 30% (Christenson et al., 2022). Hence, an urgent need exists to develop new strategies to improve on the performance of current conventional treatments.

RNA interference (RNAi), which can regulate the expression of specific genes, is regarded as a potential strategy for the treatment of a series of diseases, including cancer

therapies. Small interfering RNA (siRNA), one of RNAi therapeutics which results in sequence-specific post-transcriptional gene silencing in mammalian cell lines, has shown impressive anti-cancer potential (Elbashir et al., 2001, Evans et al., 2017). In addition, previous studies, in both *in vivo* and *in vitro* models, have shown the anti-tumor effects of siRNA in PCa therapy (Evans et al., 2017, Guo et al., 2012, Liang et al., 2022, van der Meel et al., 2021).

The tumour microenvironment (TME) is characterized as a highly complex environment surrounding tumours. The interactions between cancer cells/non-cancerous cells and cells/non-cellular components within the TME create a supporting environment for tumor initiation, development, and metastasis. Among the cell types, tumor-associated macrophages (TAMs) have gained attention due to crucial roles in supporting tumors and conferring therapy resistance (Yang et al., 2020). The immune activity of TME can be altered by reprogramming the polarization of TAMs, M2 macrophages are involved in the occurrence, development and metastasis of cancer (Cassetta and Pollard, 2018, Pathria et al., 2019). Recently immunotherapy, based on remodeling the TME and reprograming TAMs, has shown promise as a strategy for cancer treatment. The colony-stimulating factor 1/colony-stimulating factor 1 receptor (CSF-1/CSF-1R) pathway plays an important role in the polarization of TAMs. The overexpression of CSF-1 and CSF-1R on M2 macrophages is a major reason for poor tumour prognosis (Ries et al., 2015). Therefore, limiting the expression of CSF-1/CSF-1R, using siRNA, is a promising way to reprogram M2 macrophages to M1 (an anti-

tumor phenotype), the immunostimulatory phenotype, thus promoting the apoptosis of tumors (Li et al., 2020, Ries et al., 2014).

The most challenging part of siRNA-mediated reprogramming TAM is the risk of non-specific and inefficient delivery. Actively targeted drug delivery systems have been widely used in immunotherapy (Ding et al., 2021, Mi et al., 2020, Wang et al., 2020). It has been reported that sialic acid (SA) specifically targets siglec-1 (CD169) which is overexpressed on M2 macrophages with little expression in other macrophage phenotypes (Ding et al., 2021, Sui et al., 2021). Therefore, SA was selected as a targeting ligand to specifically deliver CSF-1R siRNA to M2 macrophages for PCa immunotherapy, to our knowledge this is the first time this approach has been applied to PCa.

Cyclodextrins (CDs) are cyclic oligosaccharides derived from starch, consisting of at least 6 glucose subunits linked by α -1,4 glycosidic bonds, the primary and secondary sides of the ring structure are hydrophilic and the cavity is hydrophobic (Ortiz Mellet et al., 2010, Singh et al., 2019). The hydroxyl groups on the ring structure can be modified resulting in functionalised cyclodextrins suitable for different delivery requirements. Functionalised cyclodextrins, including cationic CDs and neutral PEGylated CDs have been shown to be successful biomaterials for delivery of therapeutic nucleic acids including siRNA (Haley et al., 2020, Lai, 2014, O'Mahony et al., 2013). In addition, co-formulation of modified cyclodextrins with PEGylated lipids,

provides the opportunity to simultaneously enhance membrane permeability and attached cell specific targeting ligands (Guo et al., 2017, Sun et al., 2022, Zou et al., 2021).

In the current study, SA was conjugated with stearic acid-PEG-COOH through esterification between -COOH and -OH to form the targeting ligand, and co-formulated with an amphiphilic cationic cyclodextrin (CD) to prepare a SA-targeted CD-based nanoparticles (NPs) for the delivery of CSF-1R siRNA. The NPs were characterized and tested on both human and mouse macrophages to verify the reprogramming effects of macrophages from M2 to M1 phenotype. Subsequently, the *in vitro* anti-tumour effects were assessed using a co-culture model containing macrophages and PCa cells.

2.4 Methods and Materials

2.4.1 Cells

Human monocyte cell line THP-1 was kindly donated by Cancer Research @UCC, Ireland. Mouse macrophage cell line RAW 264.7, human PCa cell line PC-3, and transgenic adenocarcinoma of the mouse prostate cell line TRAMP C1 were purchased from American Type Culture Collection (ATCC), USA.

THP-1 cells were cultured in RPMI-1640 Medium (R8758, Sigma-Aldrich, Burlington, MA, USA) supplemented with 10% fetal bovine serum (FBS, F9665, Sigma-Aldrich, Burlington, MA, USA), 10 mM HEPES (H0887, Sigma-Aldrich, Burlington, MA,

USA), 1 mM sodium pyruvate (S8636, Sigma-Aldrich, Burlington, MA, USA), D-(+)-Glucose (G8644, Sigma-Aldrich, Burlington, MA, USA), 0.05 mM 2-Mercaptoethanol (Sigma-Aldrich, Burlington, MA, USA) and 1% Penicillin-Streptomycin (Sigma-Aldrich, Burlington, MA, USA). RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (D6429, Sigma-Aldrich, Burlington, MA, USA) supplemented with 10% FBS and 1% Penicillin-Streptomycin (P4333, Sigma-Aldrich, Burlington, MA, USA). PC-3 cells were cultured in RPMI-1640 Medium (R8758, Sigma-Aldrich, Burlington, MA, USA) supplemented with 10% FBS and 1% Penicillin-Streptomycin. TRAMP C1 cells were cultured in DMEM-high glucose (D5796, Sigma-Aldrich, Burlington, MA, USA) supplemented with 5% Nu Serum IV (355104, Corning), 5% FBS, 5 µg/mL insulin (I0516, Sigma-Aldrich, Burlington, MA, USA), 10 nM 5 α -Androstan-17 β -ol-3-one (10300, Sigma-Aldrich, Burlington, MA, USA) and 1% Penicillin-Streptomycin (Guo et al., 2012a; Guo et al., 2012b). All cells were cultured at 37 °C with 5% CO₂ and 95% relative humidity.

For THP-1 differentiation, cells were treated with 200 nM 12-O-Tetradecanoylphorbol 13-acetate (PMA, Sigma-Aldrich, Burlington, MA, USA) for 48 h to achieve differentiation from monocytes to M0 macrophages (Pantazi et al., 2021, Zheng et al., 2018). After macrophage differentiation, the media was supplemented with 20 ng/mL lipopolysaccharides (LPS, Sigma-Aldrich, Burlington, MA, USA) for 48 h to achieve differentiation from M0 to M1 macrophages (Faass et al., 2021, Liu et al., 2021), or 20 ng/mL interleukin-4 (IL-4, 200-04, PeproTech, Cranbury, NJ, USA) for 48 h to achieve

differentiation from M0 to M2 macrophages (Ma et al., 2020, Zheng et al., 2018).

For RAW 264.7 differentiation, cells were treated with 20 ng/mL LPS for 48 h to achieve differentiation from M0 to M1 macrophages (Chen et al., 2020, Sun et al., 2020), or 20 ng/mL IL-4 (214-14, PeproTech, Cranbury, NJ, USA) for 48 h to achieve differentiation from M0 to M2 macrophages (Ramesh et al., 2020, Song et al., 2018).

The expression of the sialic acid receptor CD169 (Siglec-1) was confirmed on the surface of THP-1- and RAW 264.7-derived M0/M1/M2 macrophages. Briefly, cells were differentiated as described above; after differentiation, THP-1-derived cells were stained by PE anti-human CD169 antibody (346003, BioLegend, San Diego, CA, USA); RAW 264.7-derived cells were stained by PE anti-mouse CD169 antibody (142403, BioLegend, San Diego, CA, USA) respectively. Subsequently, the expression was analyzed by Flow Cytometry.

2.4.2 Conjugation of Stearic acid-PEG-SA

Sialic acid (SA, Sigma-Aldrich, Burlington, MA, USA) and stearic acid-PEG-COOH (PG2-CASA-2k, Nanocs, New York, NY, USA) were conjugated by esterification. Briefly, SA (6 mg, 0.019 mmol, 1.5 Equiv) and stearic acid-PEG-COOH (30 mg, 0.013 mmol, 1 Equiv) were dissolved in anhydrous N,N-Dimethylformamide (1 mL, DMF, Sigma-Aldrich, Burlington, MA, USA) in the presence of N,N'-Dicyclohexylcarbodiimide (8 mg, 0.039 mmol, 3 Equiv, DCC, Sigma-Aldrich,

Burlington, MA, USA) and 4-Dimethylaminopyridine (0.476 mg, 0.0039 mmol, 0.3 Equiv, DMAP, Sigma-Aldrich, Burlington, MA, USA). The solution was heated with stirring at 60 °C for 2 days under a nitrogen atmosphere (Hu et al., 2017). Reaction completion was determined by the disappearance of stearic acid-PEG-COOH on TLC (silica: dichloromethane/methanol). The mixture was cooled and centrifuged at 15,000 rpm for 5 min to remove the insoluble by-product dicyclohexylurea. The supernatant was dialyzed against deionized water (DIW) in a Dialysis Cassette (2K MWCO, 66203, Thermo Fisher Scientific, Waltham, MA, USA) for 2 days with replacement of DIW every 12 hours to remove all other reagents and DMF. After dialysis, 14.8 mg (0.0055 mmol, 42.5 mol% based on stearic acid-PEG-COOH) of the product, stearic acid-PEG-SA, was recovered by freeze drying and subsequently stored at -20 °C. The product was identified by Fourier-transform infrared spectroscopy (Spectrum Two FT-IR Spectrometer, L160000A, PerkinElmer, Waltham, MA, USA), ¹H NMR and ¹³C NMR (Bruker Avance 600 UltraShield NMR spectrometer, Billerica, MA, USA), and High Resolution Mass Spectrometry (Waters Vion IMS Qtof, ESI, Milford, MA, USA).

2.4.3 NP Preparation

The amphiphilic cationic β -cyclodextrin (CD) was synthesized as previously described (Guo et al., 2017, O'Mahony et al., 2012). The CD was placed in a 2 mL Eppendorf tube and dissolved in chloroform. The solvent was evaporated under a stream of nitrogen so that the CD formed a thin film on the tube wall. For CD.siRNA complexation, sterilized DIW was added to rehydrate the CD, and the CD and siRNA

aqueous solution was incubated at 25 °C for 30 min with 450 rpm shaking in a ThermoMixer (Eppendorf, Germany). The untargeted and targeted NPs were formulated using a postinsertion approach by incubating the CD.siRNA complex with either 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol) (DSPE-PEG) or DSPE-PEG/stearic acid-PEG-SA at 60 °C for 60 min with 450 rpm shaking in a ThermoMixer.

2.4.4 In Vitro Characterization of the NPs

The siRNA binding with the CD in different weight ratios were examined using agarose gel electrophoresis. Briefly, free siRNA or CD.siRNA complex at weight ratios ranging from 5:1 to 20:1 containing 0.5 µg Negative Control siRNA (siNeg, Sigma-Aldrich, Burlington, MA, USA) were loaded onto a 1% (w/v) agarose gel with nucleic acid dye in Tris/Borate/EDTA buffer. Electrophoresis gels were run at 100 V for 0.5 h and imaged under a UV lamp.

Sizes and surface charges of NPs were measured by dynamic light scattering (DLS) with a Malvern Nano-ZS (Malvern Instruments, U.K.). The concentration of the CD was fixed at 0.2 mg/mL, the CD.siRNA complexes were co-formulated with DSPE-PEG at molar ratios of CD to DSPE-PEG ranging from 7:1 to 0.14:1 and measured by DLS.

The morphology of the NPs was imaged by transmission electron microscopy (TEM,

Jeol Ltd., Tokyo, Japan). Before imaging, NPs with 0.5 μg of siNeg were placed on copper grids (Agar Scientific) for 3 min and then grids were stained with 2% (w/w) uranyl acetate and left at room temperature for 12 h.

The effects of salt-containing media on the aggregation of the NPs were studied by incubating NPs in 90% OptiMEM transfection medium (Life Technologies, Carlsbad, CA, USA) for 1, 2 and 3 days at 37 °C. Then particle sizes were measured via DLS. Meanwhile, the stability of siRNA with or without CD was investigated by incubating NPs with 50% FBS for 4 and 24 h, and then analyzed via agarose gel electrophoresis.

2.4.5 Cell Culture Techniques

For competitive binding studies, THP-1- or RAW 264.7-derived M2 macrophages were cultured for 24 h, and then free SA (10 $\mu\text{g}/\text{mL}$) was added to either THP-1- or RAW 264.7-derived M2 macrophages for 60 min at 4 °C prior to the incubation of NPs coating 6-FAM-labeled negative control siRNA (100 nM, FAM-siNeg, SIC007, Sigma-Aldrich, Burlington, MA, USA). Following one more day of culture at 4 °C, both macrophages were collected and analyzed by flow cytometry (Guo et al., 2017).

To estimate cellular uptake, THP-1- or RAW 264.7-derived M2 macrophages were cultured for 1 day, and then both macrophages were transfected by targeted or non-targeted CD NPs coating 100 nM FAM-siNeg for 6 h or 24 h in serum-free media. After incubation, macrophages were washed with CellScrub Buffer (B100001, Genlatins, San

Diego, CA, USA) to totally remove all non-internalized NPs. Cells were harvested and analyzed using flow cytometry or stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged under a fluorescence microscope.

2.4.6 Cytotoxicity of the NPs

Cell viability, following exposure to the CD-based NPs, of both THP-1- and RAW 264.7-derived M2 macrophages was determined by the Cell Counting Kit-8 (CCK-8, 96992, Sigma-Aldrich, Burlington, MA, USA). THP-1- or RAW 264.7-derived M2 macrophages were cultured for 1 day. CD formulations containing 100 nM siNeg were added to M2 macrophages for 2 days. And then CCK-8 was applied to cells, and the OD450 values were measured by microplate reader (Spark®, TECAN, Switzerland). Cell viability was calculated by the following formula:

$$\text{Cell Viability (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{negative control}} - \text{OD}_{\text{blank}}} \times 100 \%$$

2.4.7 In Vitro CSF-1R Gene Silencing

The *in vitro* gene knockdown of CSF-1R was analyzed in both THP-1- and RAW 264.7-derived M2 macrophages. M2 macrophages were cultured for 1 day and transfected by free CSF-1R siRNA (siCSF-1R, human siCSF-1R, sense sequence 5'-GUU GAG ACC UUA GAG CAC ATT-3'; mouse siCSF-1R, sense sequence 5'-GGU CUU ACG CAA AAC GGU CTT-3', Thermo Fisher Scientific, Waltham, MA, USA), or different formulations containing siCSF-1R, including, CD, CD.DSPE-PEG, and CD.stearic acid-PEG-SA, or CD.stearic acid-PEG-SA coating siNeg. The concentration of siRNA

was 100 nM per well, exposure was 6 h in serum-free media and followed by replacement of complete media for another 42 h incubation under normal growth conditions. After incubation, RNA was extracted and reverse-transcribed to cDNA, and then quantitative reverse transcription PCR (RT-qPCR) was performed to evaluate the downregulation of CSF-1R mRNA in M2 macrophages. β -action was used as an internal reference (Guo et al., 2017, Schmittgen and Livak, 2008).

2.4.8 In Vitro Reprogramming of M2 Macrophages

After transfection, THP-1-derived and RAW 264.7-derived cells were harvested to analyze the expression of human (CD68) or mouse macrophage markers (F4/80), and the M1 (CD80) or M2 macrophage markers (CD206). Briefly, THP-1-derived macrophages were stained by FITC anti-human CD80 antibody (375406, BioLegend, San Diego, CA, USA), PE anti-human CD206 antibody (321106, BioLegend, San Diego, CA, USA), and APC anti-human CD68 antibody (333809, BioLegend, San Diego, CA, USA); RAW 264.7-derived macrophages were stained by PE/Cyanine7 anti-mouse CD80 antibody (104734, BioLegend, San Diego, CA, USA), FITC anti-mouse CD206 antibody (141704, BioLegend), and APC anti-mouse F4/80 antibody (142403, BioLegend, San Diego, CA, USA). The cells were then measured by Flow cytometry.

2.4.9 Cell Co-Culture Techniques

A Transwell model (3 μ m pore size, 662630, Greiner Bio-One, Stonehouse, U.K.) was

built by placing 5.0×10^4 M2 macrophages in the upper chamber (100 μ L per well) and 1.0×10^5 PCa cell in the lower well (600 μ L per well) (Li et al., 2020). Both human cells, THP-1-derived M2 macrophages/PC-3 cells, and mouse cells, RAW 264.7-derived M2 macrophages/TRAMP-C1 cells, were used. Free siCSF-1R, CD, CD.DSPE-PEG, and CD.stearic acid-PEG-SA coating siCSF-1R, and CD.stearic acid-PEG-SA coating siNeg were applied on the upper chambers for 2 days. The concentration of siRNA used was 100 nM. After treatment, the supernatant in the lower well was collected. The M1 cytokine interleukin-12 (IL-12) and the M2 cytokine interleukin-6 (IL-6) were measured by ELISA (human IL-12 kit, 431707, BioLegend; mouse IL-12 kit, 433607, BioLegend; human IL-6 kit, 430507, BioLegend; mouse IL-6 kit, 431307, BioLegend, San Diego, CA, USA). The total RNA of macrophages in the upper chambers were extracted to measure M1 factors, including IL-12, CXCL-10, and TNF- α , and M2 factors, including IL-6, IL-10, Arg-1, and CD163 by RT-qPCR.

To explore if the reprogramming of M2 macrophages induced PCa cells apoptosis, the apoptosis of PC-3 cells and TRAMP-C1 cells was analyzed by FITC Annexin-V Apoptosis Detection Kit with PI (640914, BioLegend, San Diego, CA, USA) via flow cytometry. Briefly, after incubation with the different formulations for 48 h, the PCa cells in the lower wells were collected, stained with FITC-Annexin V and Propidium Iodide (PI) and analyzed by flow cytometry.

2.4.10 Statistical analysis

The statistical analysis was performed using Prism 8.0 (GraphPad, San Diego, CA, USA). Data were shown as mean $\pm$ standard deviation (SD) from at least 3 independent experiments. One-way analysis of variance (one-way ANOVA), followed by Dunnet or Tukey post hoc tests and Student's t-test was used to calculate the statistical significance between three or more groups and two groups. A p-value lower than .05 was considered a significant difference.

2.5 Results and Discussion

2.5.1 Preparation and Characterization of CD NPs

Stearic acid-PEG-SA was successfully synthesized, and the structure was confirmed by FT-IR, $^1\text{H}/^{13}\text{C}$ NMR, and Mass spectrometry (Fig. 2.1).

Fourier Transform Infra-Red (Spectrum Two FT-IR Spectrometer, L160000A, PerkinElmer, Waltham, MA, USA): Fig. 2.1B shows FT-IR spectra of starting materials SA, stearic acid-PEG-COOH and the product stearic acid-PEG-SA. Table 1. identifies the key FT-IR absorptions for each compound. Stearic acid-PEG-SA is seen to exhibit absorptions associated with an ester link, amide I & II and PEG functionality. The carboxylic acid group is likely to overlap with the ester absorption (1699cm^{-1}) due to changes in internal hydrogen bonding driven by addition of the large PEG component of the conjugate. O-H stretching absorption (3268cm^{-1}) is seen to be weak in the conjugate spectrum relative to the starting materials as O-H functionality is proportionally lower in the conjugate and the carboxylic acid contribution (relative to

the parent molecules) is reduced via esterification.

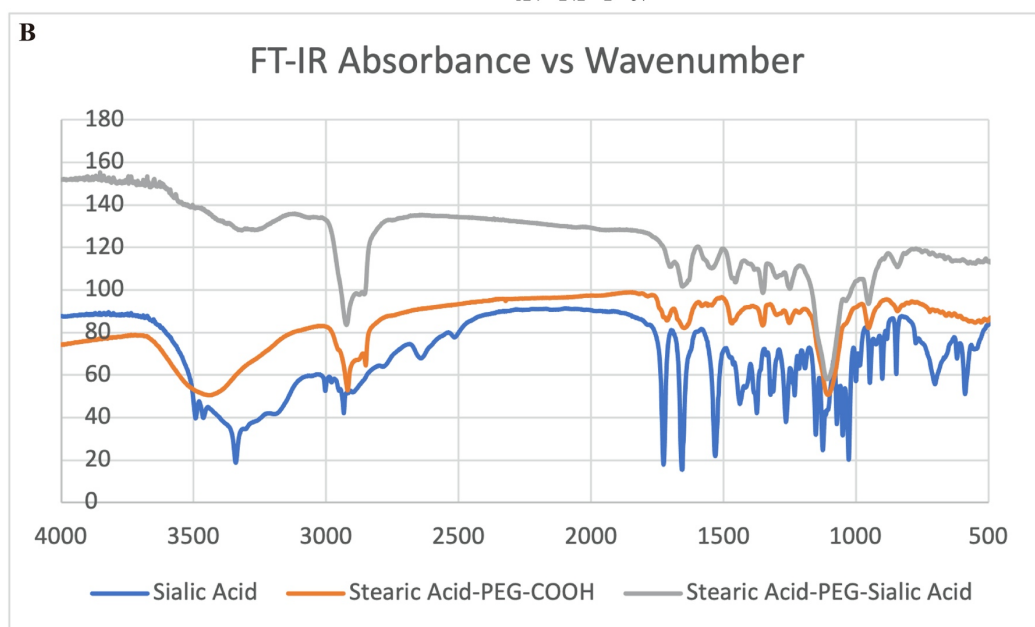
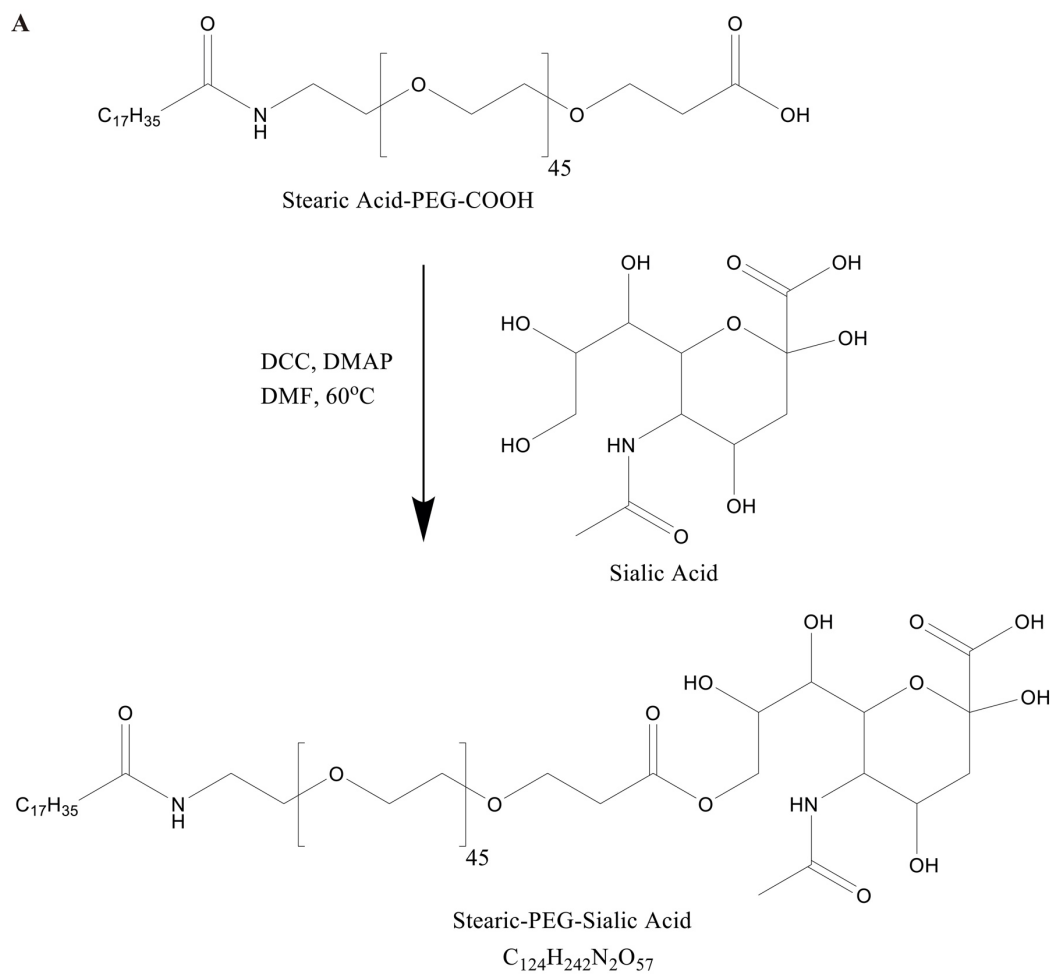
Table 1. The key FT-IR absorptions for each compound

Functional Group	Stearic acid-PEG-COOH /cm ⁻¹	Sialic Acid /cm ⁻¹	Stearic Acid-PEG-SA /cm ⁻¹
Carboxylic Acid	1714	1726	1699
Ester	N.A.	N.A.	1699
Amide I & II	1641 / 1548	1657 / 1531	1655 / 1542
PEG (C-O-C)	1103	N.A.	1103
O-H stretch	3434	3304	3268 (weak)

High Resolution Mass Spectrometry (Waters Vion IMS Qtof, ESI, Milford, MA, USA) also confirms the conjugation: calculated for C₁₂₄H₂₄₅N₂O₅₇ [M+3H]⁺: 2674.6334, found: 2674.6340. Error: 0.85 ppm. This molecular formula corresponds to a conjugate containing 45 glycol units (PEG2k). The product contains a range of conjugates which differ slightly in the number of glycol units.

The proton NMR spectrum (Bruker Avance 600 UltraShield NMR spectrometer, Billerica, MA, USA), Fig. 2.1C contains resonances to confirm the presence of stearic (0.85 ppm, 1.23 ppm), PEG (3.51 ppm) and SA (1.5 - 2.0 ppm, 8.27ppm) residues in the conjugate.

^{13}C NMR, Fig. 2.1C indicates the presence of five carbonyl species (153.94, 157.06, 171.80, 172.62, 178.06ppm). The conjugate contains two amide groups, one ester link and one carboxylic acid group. However, the proton and carbon spectra (Otsuka et al., 2003) are complicated by the existence of α and β isomers of the SA residue; hence the presence of an additional signal is not surprising.



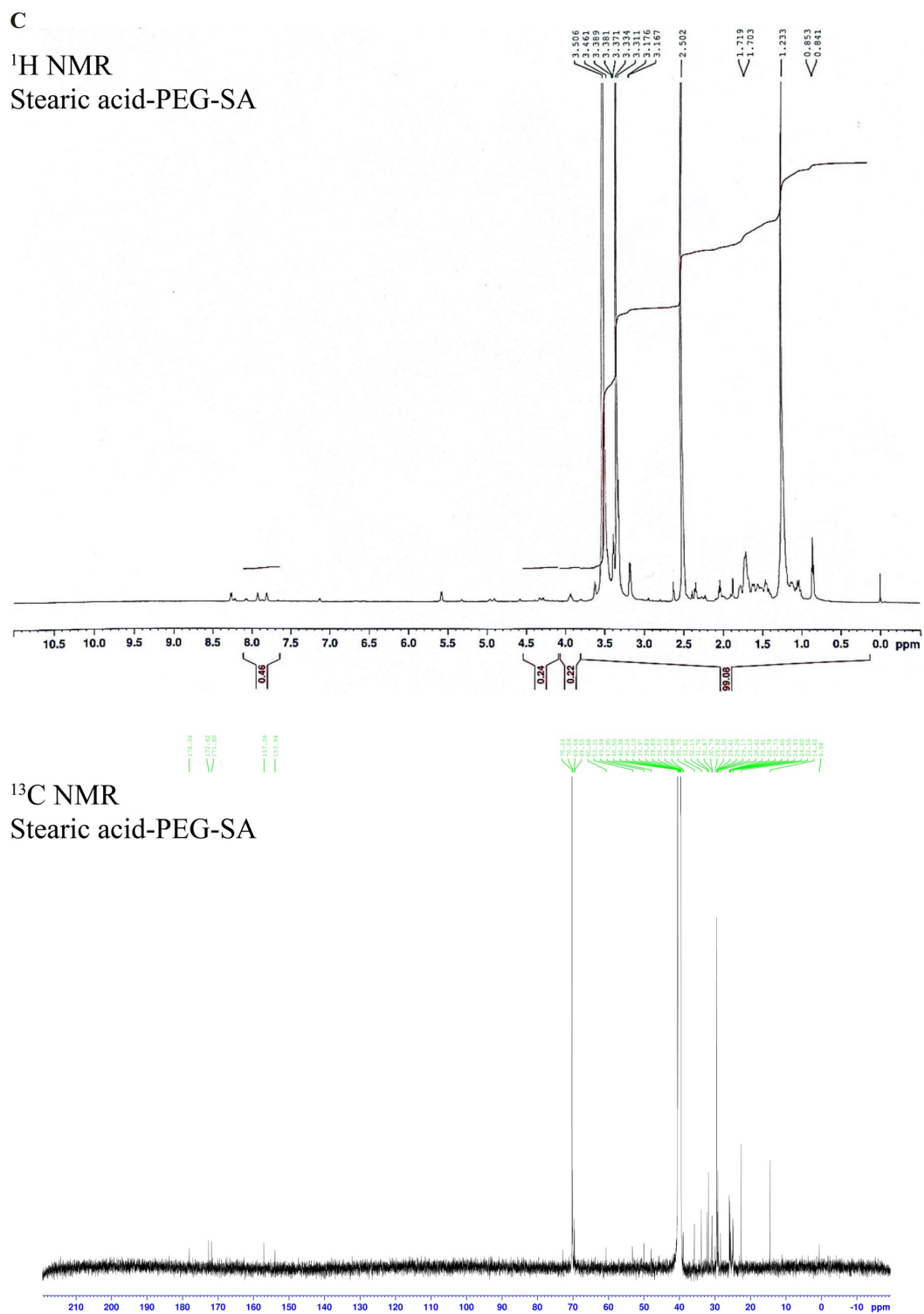


Fig. 2.1. (A) Synthesis of stearic acid-PEG-SA. (B) FT-IR spectra of SA, stearic acid-PEG-COOH, and stearic acid-PEG-SA. (C) ¹H NMR spectrum of stearic acid-PEG-SA; and ¹³C NMR spectrum of stearic acid-PEG-SA.

To screen the optimal weight ratio of CD to siRNA, CD.siRNA formulations in a range of WR 5 to WR 20 were imaged on agarose gel, results show no free siRNA, indicating full binding by the CD formulations (Fig. S2.1). This result is consistent with our previous published data showing that the CD fully complexes the siRNA from WR 5 and above (Godinho et al., 2013). As indicated by DLS, CD.siRNA (WR 10) formed a nanocomplex of 151 ± 3 nm, a surface charge of 32 ± 1 mV with the lowest PDI (< 0.3) of the three formulations (Table. S1). Therefore, the WR 10 of CD.siRNA was used for all subsequent work.

DSPE-PEG/stearic acid-PEG-SA was incorporated into CD formulations by a postinsertion approach (Fig. 2.2). Stearic acid is an 18-carbon saturated fatty acid lipid. As the lipid structure is similar to DSPE, the same postinsertion approach to incorporation of the targeting ligand (stearic acid-PEG-SA) was achieved (Guo et al., 2017).

To optimize the molar ratio between CD and DSPE-PEG, a range of 7:1 to 0.14:1 was measured by DLS. A 0.35:1 molar ratio of CD to DSPE-PEG, displayed optimum size (206 nm), PDI (< 0.2), and charge (19 mV) compared with other ratios (Table. S2).

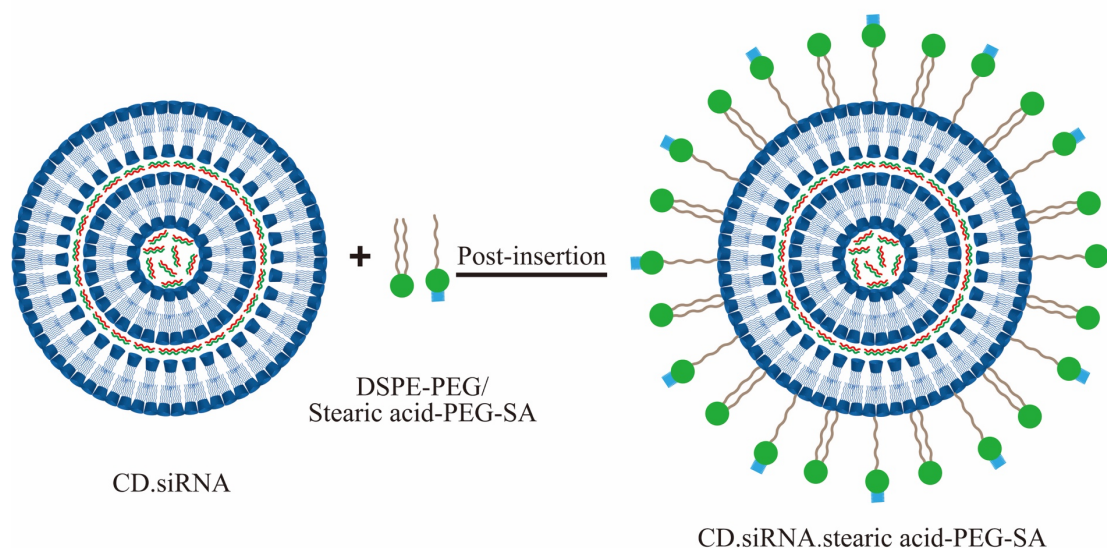


Fig. 2.2. A schematic of the formulation of the M2 macrophage-targeting NP CD.siRNA.stearic acid-PEG-SA prepared by incorporating the ligand stearic acid-PEG-SA into CD.siRNA formulation using a post-insertion approach.

In the targeted formulation, CD.siRNA.stearic acid-PEG-SA, the weight ratio of CD to siRNA was 10, the molar ratio of CD to DSPE-PEG to stearic acid-PEG-SA was 0.35: 0.5: 1. Agarose gel imaging showed no free siRNA in all CD formulations (Fig. 2.3A), suggesting that the incorporation of DSPE-PEG or stearic acid-PEG-SA did not affect the binding of CD and siRNA. The size and charge of the targeted NPs (CD.siRNA.stearic acid-PEG-SA) were 246 ± 16 nm, and 29 ± 2 mV respectively, indicating an increase in size and charge ($*p < 0.05$) compared with the non-targeted NPs (CD.siRNA.DSPE-PEG formulations) (Fig. 2.3B & Table. S3). The increase in size suggests the successful incorporation of stearic acid-PEG-SA.

All three CD-based formulations, imaged by TEM, were spherical. The CD.siRNA NPs displayed a solid structure, similar to our previous results (Evans et al., 2016, Guo et al., 2017). The halo-like structure surrounding the surface of the CD.siRNA.DSPE-PEG NPs may indicate a protective layer of PEG. This is also consistent with our previous results (Guo et al., 2017, O'Mahony et al., 2013). In contrast, the morphology of the CD.siRNA.stearic acid-PEG-SA NPs varied slightly compared to both the CD.siRNA or CD.siRNA.DSPE-PEG formulations due possibly to the presence of the SA ligand in the steric acid-PEG-SA (Fig. 2.3C).

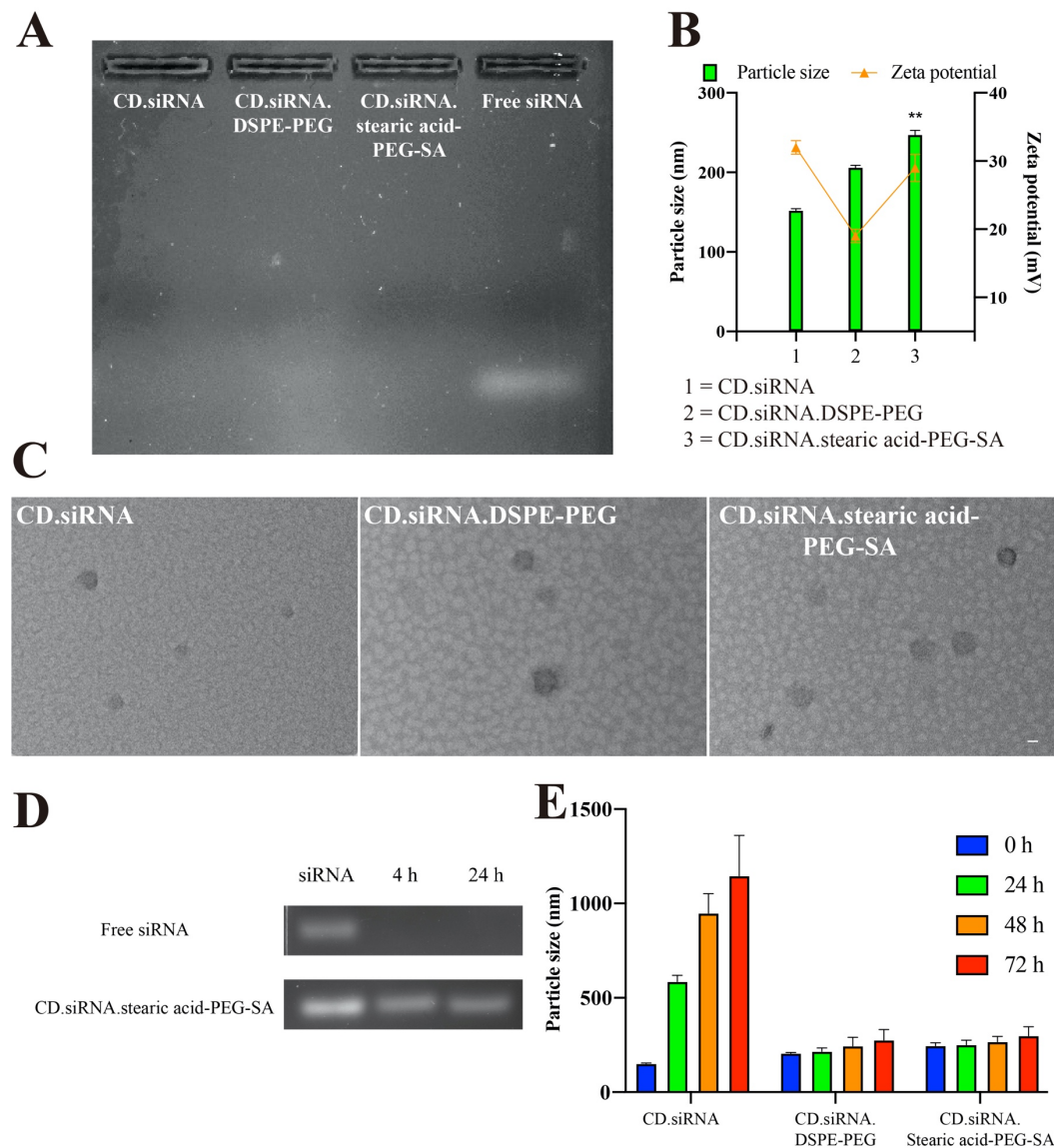


Fig. 2.3. Physicochemical characterization of CD NPs. (A) Complexation of siRNA (0.5 μ g) with CD NPs. (B) Sizes and surface charge of NPs ($n = 6$, mean $\pm$ SD, $**p < 0.01$, versus CD.siRNA.DSPE-PEG) (C) Morphology of CD NPs (scale bar = 100 nm). (D) The stability of free siRNA and targeted NPs in serum-containing media was analyzed by agarose gel electrophoresis. (E) Size changes of CD NPs in salt-containing media for 1, 2, and 3 days ($n = 6$, mean $\pm$ SD).

The ability of the CD.stearic acid-PEG-SA NPs to protect against RNA degradation in

serum-containing media was assessed. Compared to the siRNA control, following incubation in 50% FBS for up to 24 h, the targeted NPs protected the siRNA from degradation as indicated by the heparin induced release of intact cargo (Fig. 2.3D). Size changes of 3 CD NPs in salt-containing media in 3 days were measured by DLS every 24 h (Fig. 2.3E). Compared with non-PEGylated NPs, the PEGylated NPs avoided aggregation, suggesting that the incorporation of PEG enhanced the stability of the NPs.

2.5.2 Cellular Uptake by M2 macrophages

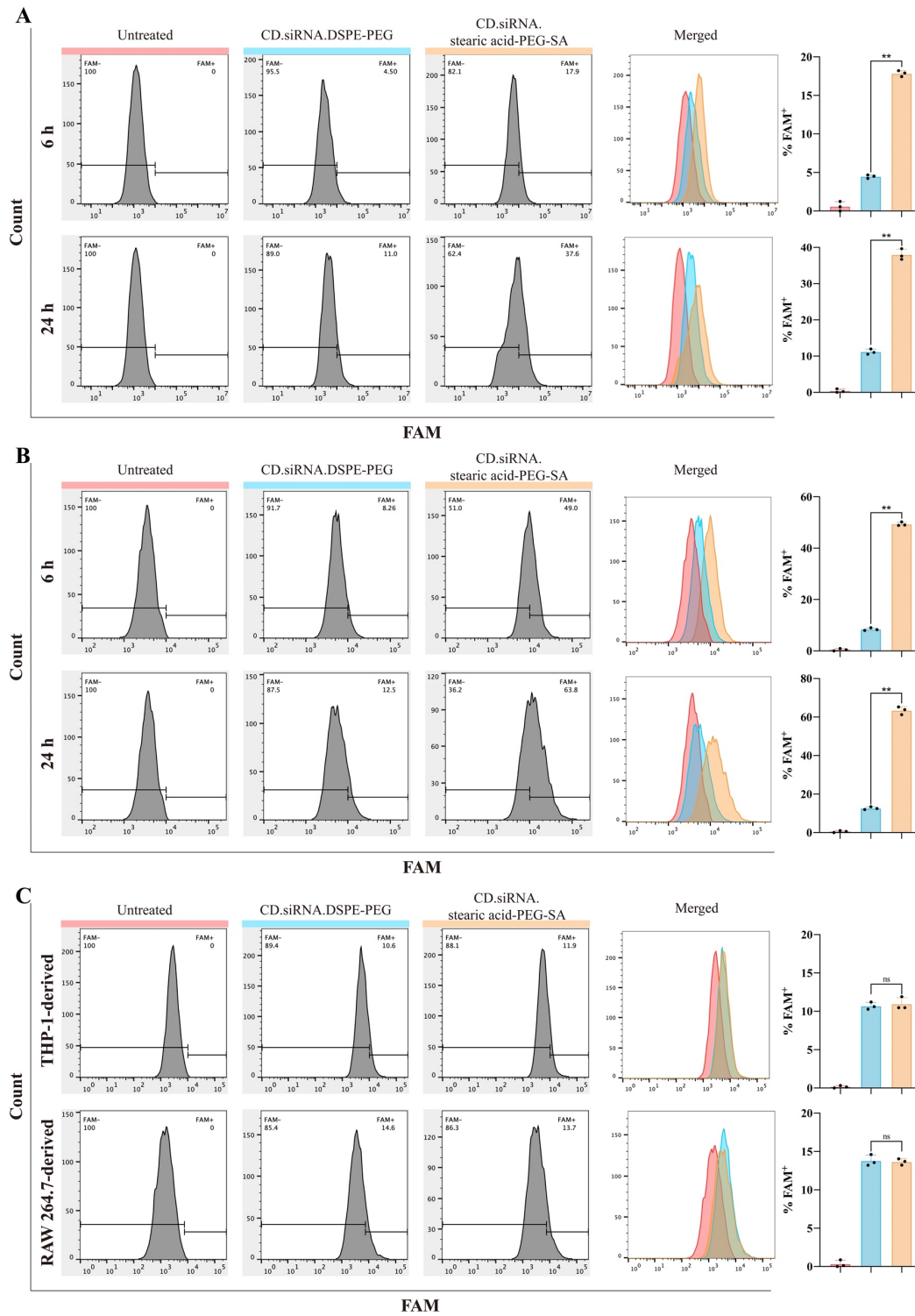
The expression of the SA receptor, CD169 (Siglec-1) on different phenotypes of macrophages was analyzed by flow cytometry, results showed that CD169 was overexpressed by M2 macrophages (65.5% CD169⁺ on THP-1-derived M2 macrophages; 72.6% CD169⁺ on RAW 264.7-derived M2 macrophages) with little expression by M0 or M1 macrophages (< 1% CD169⁺, Fig. S2.2).

The uptake of the targeted versus the non-targeted formulation by M2 Macrophages was compared using FAM-labelled siRNA. For THP-1-derived M2 macrophages, 17.9% and 37.6% (n = 3, ***p* < 0.01) of cells were FAM⁺ in the targeted formulation group at 6 h and 24 h post-incubation, compared with 4.5% and 11.0% in the non-targeted formulation group, respectively (n = 3, ***p* < 0.01, Fig. 2.4A). Similar results were observed with RAW 264.7-derived macrophages, where FAM⁺ cells were 49.0% and 63.8% in the targeted formulation group at 6 h and 24 h post-incubation (n = 3, ***p* < 0.01, Fig. 2.4B). These results were confirmed by fluorescent microscopy (Fig. 2.4D-

E), more FAM-siNeg (green) was observed in the targeted NPs group in the case of both THP-1- and RAW 264.7-derived M2 macrophages after 6 h or 24 h transfection. It has been reported that SA-targeted formulations increased cellular uptake in RAW 264.7-derived M2 macrophages by 2.53 fold compared to the non-targeted formulations (Li et al., 2021). In the current study, a similar trend was observed where the SA-targeted CD formulations increased cellular uptake in THP-1-derived M2 macrophages by 4.00 and 3.40 times after 6 h and 24 h transfection, and in RAW 264.7-derived M2 macrophages by 5.86 and 5.02 times after 6 h and 24 h transfection respectively.

To confirm that the higher cellular uptake of the targeted formulation was associated with the SA-based ligand, a competitive uptake study was performed. Results showed that following pre-treatment with free SA, no difference between the targeted and non-targeted formulations was observed in THP-1- (11.9% vs 10.6% FAM+, $n = 3$, $p = 0.6629$) or RAW 264.7-derived M2 macrophages (Fig. 2.4C, 13.7% vs 14.6% FAM+, $n = 3$, $p = 0.7967$), indicating that the higher cellular uptake in the targeted formulation group was related to the presence of the SA targeting ligand.

This is consistent with the results in the literature, where Siglec-1 overexpressed in M2 macrophages mediated the targeting of a SA conjugate to macrophages (Li et al., 2021).



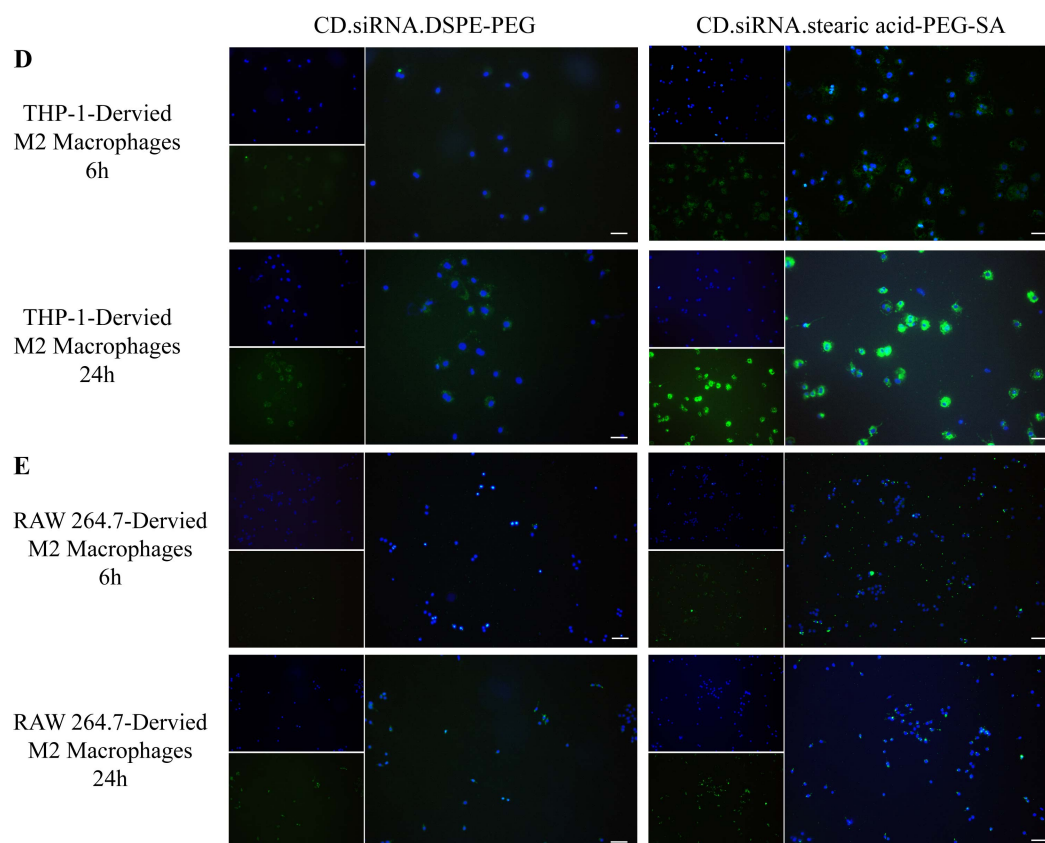


Fig. 2.4. *In vitro* characterization of CD NPs. Cellular uptake of non-targeted and targeted NPs in THP-1- (A) and RAW 264.7-derived M2 macrophages (B) after 6 or 24 h incubation. ($n = 3$, mean $\pm$ SD, $**p < 0.01$). For the competitive binding study (C), free SA was added to THP-1- and RAW 264.7-derived M2 macrophages prior to the addition of NPs. Non-targeted and targeted NPs were then incubated with cells for 24 h, $n = 3$, $**p < 0.01$. Fluorescent microscopic images of cellular uptake of targeted and non-targeted NPs in THP-1- (D) and RAW 264.7-derived M2 macrophages (E) after 6 h or 24 h transfection (Blue: DAPI, Green: FAM-siNeg, scale bar = 200 μm).

2.5.3 *In Vitro* Remodeling of TAMs

To evaluate the cytotoxicity of the CD NPs, M2 macrophages were incubated with a negative control siRNA (siNeg, 100 nM) formulations for 48 h and the cell viability

was measured by the CCK-8 method. For all three CD formulations, CD.siNeg, CD.siNeg.DSPE-PEG, and CD.siNeg.stearic acid-PEG-SA no significant toxicity was detected relative to the untreated control ($n = 3$, $p = 0.6086$, 0.7620 , 0.3916 in THP-1-derived M2 macrophages, and $p = 0.9976$, 0.8554 , 0.1099 in RAW 264.7-derived M2 macrophages, Fig. S2.3).

To investigate the remodeling of M2 to M1 macrophages, the *in vitro* downregulation of CSF-1R was analyzed in both human and mouse M2 macrophages. The targeted formulation, CD.siRNA.stearic acid-PEG-SA, significantly decreased the expression of CSF-1R mRNA (by $29.64\% \pm 1.66\%$ in THP-1-derived M2 macrophages; $38.94\% \pm 0.97\%$ in RAW 264.7-derived M2 macrophages) compared with the other groups (Fig. 2.5A-B, $**p < 0.01$). In addition, although the gene silencing efficacy of the non-targeted formulations ($19.31\% \pm 1.21\%$ in THP-1-derived M2 macrophages; $18.51\% \pm 2.85\%$ in RAW 264.7-derived M2 macrophages) were significantly lower than the targeted NPs, they were still significantly different relative to the other remaining groups ($**p < 0.01$).

In addition, the expression of the human or mouse macrophage marker, CD68 or F4/80, and M1 or M2 macrophages markers, CD86 or CD206 respectively, were measured in the post-transfected THP-1-derived M2 macrophages. Following treatment with the targeted formulation, 54.2% of cells expressed CD86, and $\sim 44.4\%$ expressed CD206, suggesting that over 50% of THP-1-derived M2 macrophages were reprogrammed by

the CD.siRNA.stearic acid-PEG-SA formulation (Fig. 2.5C & E). A similar result was observed in RAW 264.7-derived M2 macrophages, where 67.5% of cells expressed CD86 while 35.9% of cells expressed CD206, suggesting that over 60% of M2 macrophages were reprogrammed by the targeted NPs (Fig. 2.5D & F). The expression of M1/M2 markers was consistent with gene knockdown results.

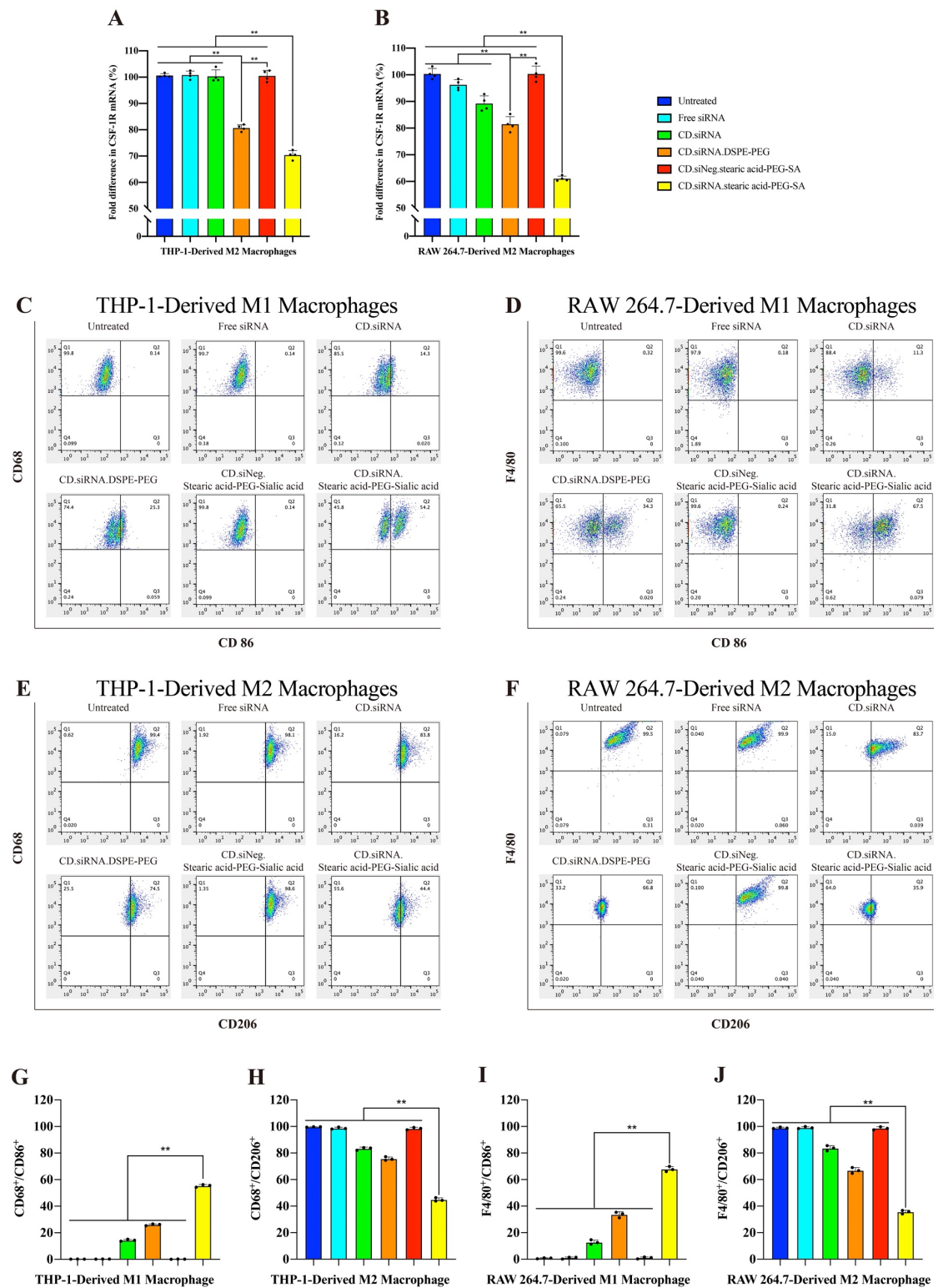


Fig. 2.5. *In vitro* reprogramming of TAMs. *In vitro* gene knockdown of CSF-1R on THP-1- (A) and RAW 264.7-derived M2 macrophages (B). Cells were incubated with Free siRNA, CD.siRNA, CD.siRNA.DSPE-PEG, CD.siNeg.stearic acid-PEG-SA, and CD.siRNA.stearic acid-PEG-SA for 6 h, and for another 42 h culture under normal

condition, mRNA were extracted, then reverse transcription and RT-PCR were performed. GAPDH was used as internal reference (mean $\pm$ SD, $n = 6$, ** $p < 0.01$). After transfection, the expression of the human macrophage marker CD68 and the M1/M2 macrophage markers CD86 (C)/CD206 (D) were measured on the THP-1-derived macrophages; and the mouse macrophage marker F4/80 and the M1/M2 macrophage markers CD86 (E)/CD206 (F) were measured on the RAW 264.7-derived macrophages by Flow Cytometry. Percentage M1 macrophages in THP-1- (CD68+/CD86+, G) and RAW 264.7-derived macrophages (F4/80+/CD206+, I), and M2 macrophages in THP-1- (CD68+/CD206+, H) and RAW 264.7-derived macrophages (F4/80+/CD206+, J) based on the FACS results ($n = 3$, ** $p < 0.01$).

In summary, the results above indicate that the M2 macrophages were reprogrammed to the M1 phenotype through the downregulation of the CSF-1R gene, which stems from the successful delivery and silencing of CSF-1R siRNA in M2 macrophages, the response was significantly ($p < 0.01$) stronger with the SA targeted NP formulation as mediated via the SA-CD169 axis.

Other studies with CSF-1R siRNA-loaded polyethylenimine-stearic acid-based micelles and an M2 peptide-targeting ligand have achieved similar levels of TAM reprogramming, resulting in 40% knockdown of CSF-1R mRNA in M2 macrophages (Li et al., 2020). Nanoparticles formulated with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and cholesterol oleate (C.O) and loaded with CSF-1R siRNA

also achieved similar CSF-1R gene knockdown (Qian et al., 2017). In addition, it has been reported that nanoparticles based on a cationic nanohydrogel containing CSF-1R siRNA with α -mannose as the ligand specifically targeted M2 polarized primary macrophages to knockdown the expression of CSF-1R level and reprogram M2 macrophages (Leber et al., 2019). These results indicate the potential of TAM reprogramming by downregulation of CSF-1R in cancer immunotherapy.

2.5.4 In Vitro Anti-Tumour Effects

The *in vitro* anti-tumour effects were analyzed using a Transwell co-culture model, where M2 macrophages and PCa cells were seeded in the upper and lower chambers respectively. After incubation, the M1/M2 factor mRNA levels were measured in the M2 macrophages. In the human macrophages, M1 factors (IL-12, CXCL10, and TNF- α) were significantly upregulated by the targeted formulation (Fig. 2.6A - C, $**p < 0.01$) while mRNA levels of M2 factors (IL-6, IL-10, Arg-1, and CD163) were downregulated by the targeted NPs (Fig. 2.6D - G, $*p < 0.05$; $**p < 0.01$). In mouse macrophages, similar results were observed, with upregulation of M1 cytokines and downregulation of M2 cytokines following treatment with the CD.siRNA.stearic acid-PEG-SA NPs (Fig. 2.6H - N, $*p < 0.05$; $**p < 0.01$).

In addition, the ELISA was performed to measure the protein levels of the M1 cytokine, IL-12, and M2 cytokine, IL-6 in the lower chamber of the Transwell model. For both PC-3 and TRAMP C1 cells, the level of IL-6 was decreased, while the level of IL-12

was increased by the targeted formulation (Fig. 2.6O - R, $*p < 0.05$; $**p < 0.01$), which is in accordance with the qPCR results for the mRNA levels of these cytokines. These results indicate that the targeted NPs could reprogram M2 macrophages, encourage the secretion of M1 cytokines and limit the secretion of M2 cytokines.

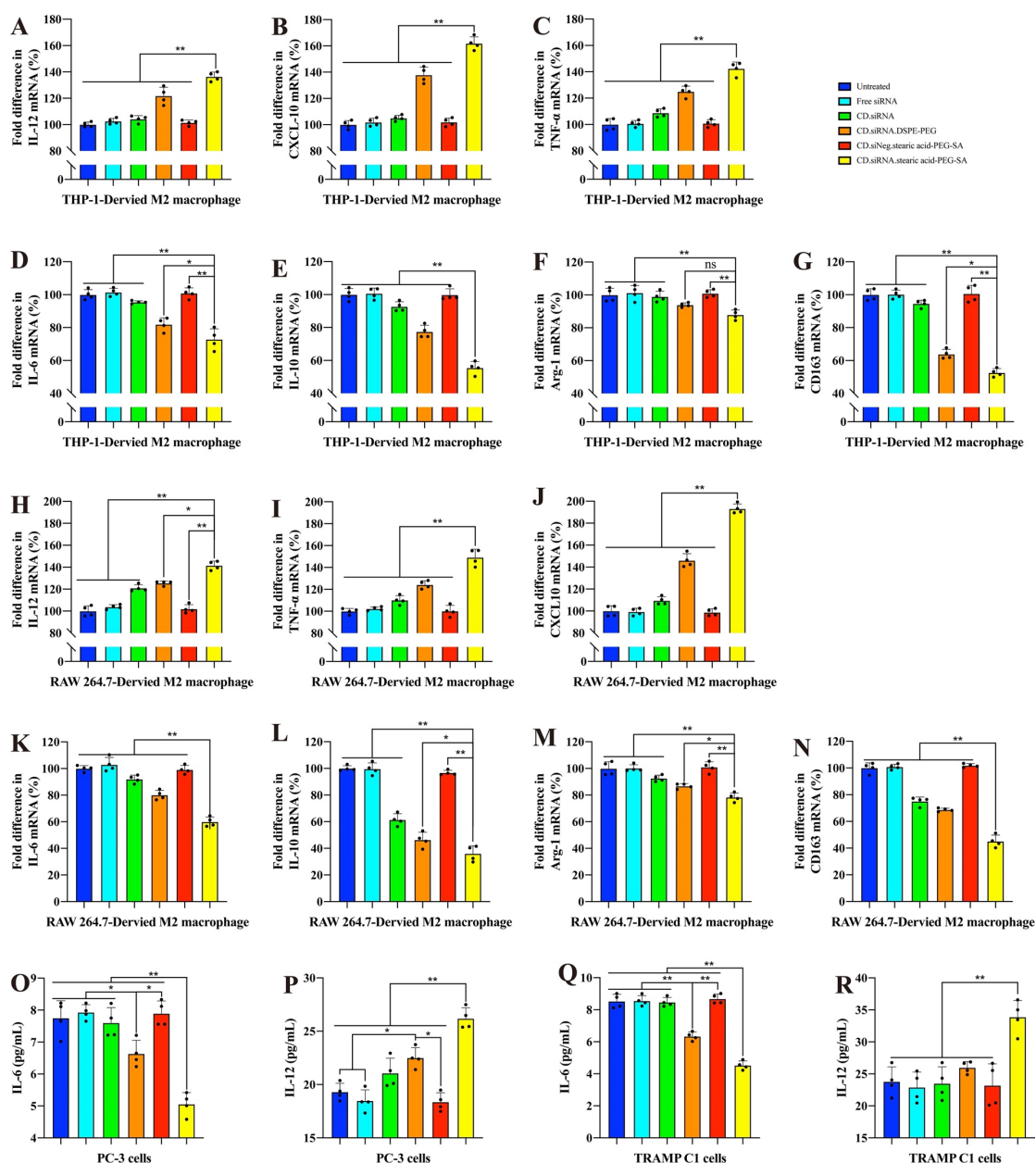


Fig. 2.6. *In vitro* anti-tumour effects. RT-qPCR results of M1/M2 factors production in THP-1-derived macrophages: (A) IL-12, (B) CXCL 10, (C) TNF- α , (D) IL-6, (E) IL-

10, (F) Arg-1, and (G) CD163; and in RAW 264.7-derived macrophages: (H) IL-12, (I) CXCL 10, (J) TNF- α , (K) IL-6, (L) IL-10, (M) Arg-1, and (N) CD163 (n = 4, mean $\pm$ SD, * p < 0.05, ** p < 0.01, ns = not significant). ELISA results of cytokine productions in PC-3 cells supernatant: (O) IL-6, and (P) IL-12; and in TRAMP C1 cells supernatant: (Q) IL-6, and (R) IL-12 (n = 4, mean $\pm$ SD, * p < 0.05, ** p < 0.01).

As a further indicator of anti-tumour effects resulting from the reprogramming of TAMs, the level of apoptosis of prostate cancer cells in the lower chamber was measured by Annexin V/PI double stained. Treatment with the targeted formulation resulted in 49.17% $\pm$ 4.78% apoptosis in PC-3 cells, significantly higher relative to the other groups, (Fig. 2.7A & C, n = 3, ** p < 0.01), and 69.15% $\pm$ 4.76% apoptosis in TRAMP C1 cells (Fig. 2.7B & D, n = 3, ** p < 0.01). In summary, the targeted delivery of CSF-1R siRNA successfully reprogrammed M2 macrophages, and the reprogrammed M1 macrophages released M1 factors, including cytokines and chemokines, which inhibited prostate cancer cell growth.

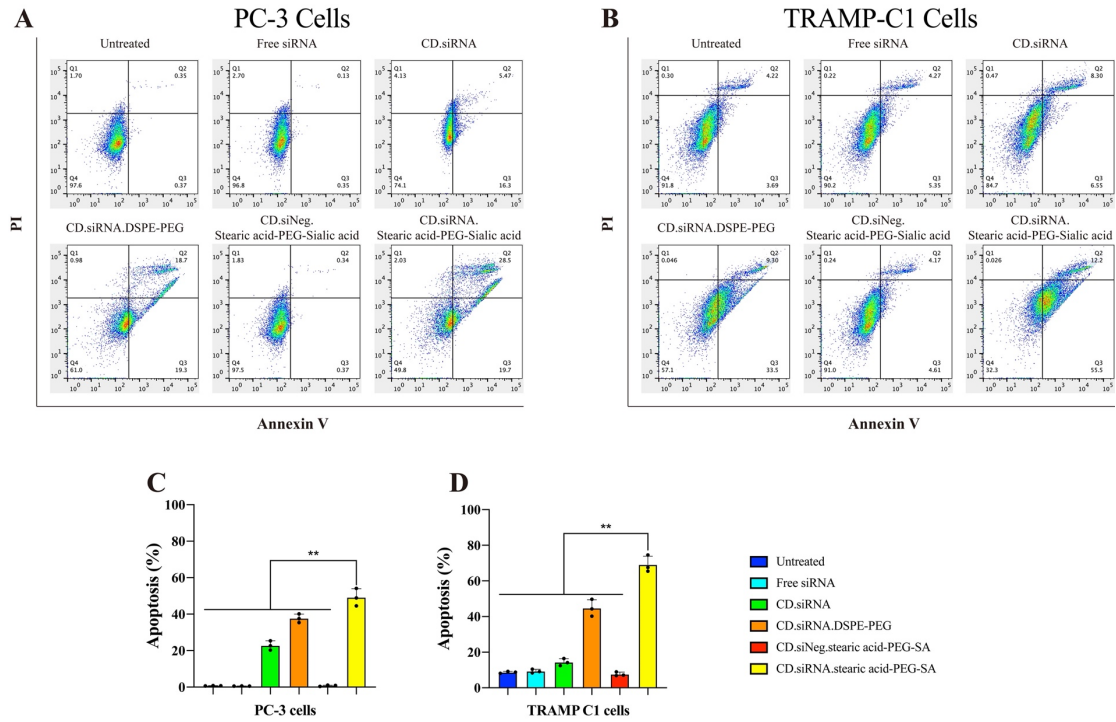


Fig. 2.7. Apoptosis of PCa cells in the lower chambers of Transwell plates. (A) PC-3 cells and (B) TRAMP C1 cells. Percentage of apoptosis of (C) PC-3 cells and (D) TRAMP C1 cells based on the FACS results (n = 3, mean $\pm$ SD, ** p < 0.01).

The above results clearly show when TAMs are reprogrammed into M1 macrophages, prostate cancer immunotherapy is achieved by up-regulating the release of M1 cytokines and down-regulating M2 cytokines. While this approach, silencing CSF-1R via siRNA, has been explored for pancreatic cancer and melanoma tumour treatment (Li et al., 2020, Qian et al., 2017), to our knowledge this is the first time it has been applied to prostate cancer.

In the case of pancreatic cancer an M2 peptide-targeted CSF-1R siRNA-loaded polyethylenimine-stearic acid-based micelles achieved ~40% apoptosis of pancreatic

cancer Pan-02 cells as detected using a co-culture model (Li et al., 2020). Similar to pancreatic cancer, prostate cancer is an immunosuppressive tumour (Stultz and Fong, 2021), due to the high infiltration of regulatory T cells (Treg), TAMs, and myeloid-derived suppressor cells (MDSCs), and the dense fibrotic tumour micro-environment. All these factors lead to a high level of immune checkpoints and low level of cytotoxic T cells infiltration. Therefore, the development of new immunotherapeutic approaches is a promising therapeutic strategy for the treatment of prostate cancer.

The positive transfection efficiency and the resulting anti-tumour effects suggest that this targeted CD-based NPs has potential to be a novel immunotherapeutic strategy for prostate cancer. In addition, opportunities exist to further enhance the efficacy using a combination therapy approach by co-delivery of the siRNA NP with a chemotherapeutic agent (Li et al., 2020). The ability of CD to form inclusion complexes with small poorly water soluble drugs, such as many anti-cancer therapeutics including ginsenoside Rg3, quercetin, and docetaxel, is an additional benefit of this formulation approach (Zou et al., 2021, Sun et al., 2022).

2.6 Conclusion

Prostate cancer is difficult to manage with conventional treatments, consequently new therapeutic strategies designed to exploit the tumour microenvironment are urgently needed. Immunotherapy has recently shown positive clinical results in a range of cancers therefore the application of this approach to prostate cancer, an

immunosuppressive tumour, is worthy of investigation. In this work a targeted NP was formulated to deliver siRNA to silence colony stimulating factor-1 receptor. The flexibility of the modified amphiphilic cationic CD biomaterial simultaneously facilitated the complexation of the siRNA and the successful incorporation of the targeting ligand, sialic acid. The resulting NPs displayed acceptable physicochemical properties consistent with high levels of selective uptake into the targeted macrophages resulting in reprogramming of M2 macrophages to the immunostimulatory M1 phenotype. The ability of the targeted CD-based NPs to enhance apoptosis in PCa and the additional future capacity to co-deliver with a suitable chemotherapeutic drug indicates the therapeutic potential of this formulation approach.

2.7 CRediT authorship contribution statement

Yao Sun: Methodology, Formal analysis, Writing – original draft. Michael F. Cronin: Methodology, Formal analysis, Writing – review & editing. Monique C.P. Mendonça: Resources, Writing – review & editing. Jianfeng Guo: Conceptualization, Supervision, Writing – review & editing. Cairiona M. O’Driscoll: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

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2.9 Supporting Information

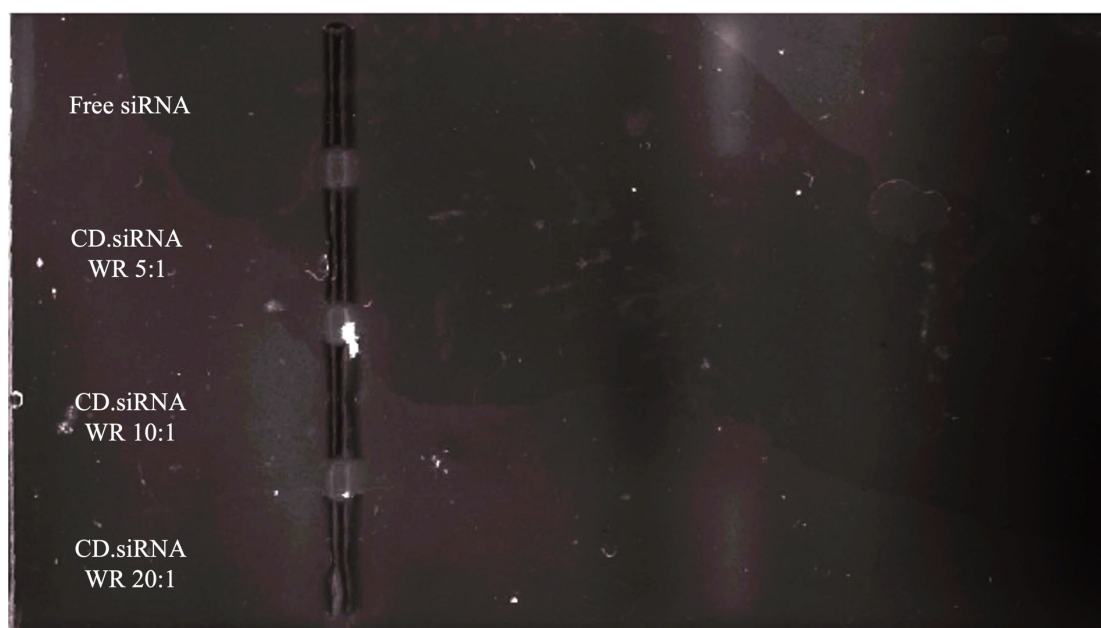


Fig. S2.1. Agarose gel results of CD.siRNA formulations in a range of WR 5 to WR 20.

Table S1. Particle size and zeta potential of CD.siRNA formulations [at various weight ratios (WRs) of CD to siRNA] measured in deionized water using DLS. (PDI = polydispersity index)

Formulation	Size (nm)	PDI	Zeta potential (mV)
CD.siRNA (WR 5)	246 $\pm$ 3	0.28	34 $\pm$ 1
CD.siRNA (WR 10)	151 $\pm$ 3	0.26	32 $\pm$ 1
CD.siRNA (WR 20)	214 $\pm$ 21	0.39	30 $\pm$ 3

n = 6, mean $\pm$ SD

Table S2. Particle size and zeta potential of CD.siRNA.DSPE-PEG formulations (WR 10) [at various molar ratios (MRs) of CD to DSPE-PEG] measured in deionized water using DLS.

Formulation	Size (nm)	PDI	Zeta potential (mV)
CD.siRNA.DSPE-PEG (MR 7)	201 $\pm$ 5	0.28	28 $\pm$ 4
CD.siRNA.DSPE-PEG (MR 3.5)	196 $\pm$ 13	0.23	25 $\pm$ 3
CD.siRNA.DSPE-PEG (MR 0.7)	257 $\pm$ 6	0.25	22 $\pm$ 2
CD.siRNA.DSPE-PEG (MR 0.35)	206 $\pm$ 4	0.17	19 $\pm$ 1
CD.siRNA.DSPE-PEG (MR 0.14)	310 $\pm$ 10	0.47	5 $\pm$ 2

n = 6, mean $\pm$ SD

Table S3. Particle size and zeta potential of CD formulations measured in deionized water using DLS.

Formulation	Size (nm)	PDI	Zeta potential (mV)
CD.siRNA	151 $\pm$ 3	0.28	32 $\pm$ 1

CD.siRNA.DSPE-PEG	206 ± 4	0.17	19 ± 1
CD.siRNA.stearic acid-PEG-SA	246 ± 16	0.27	29 ± 2

n = 6, mean ± SD

Table S4. Preparation of CD and siRNA formulations. (WR = weight ratio; MR = molar ratio) (siRNA = CSF-1R siRNA; siNeg = Non silencing siRNA)

Formulation	Preparation
CD.siRNA	WR of CD to siRNA = 10
CD.siRNA.DSPE-PEG	WR of CD to siRNA = 10 MR of CD to DSPE-PEG = 0.35
CD.siRNA.stearic acid-PEG-SA	WR of CD to siRNA = 10 MR of CD to DSPE-PEG = 0.7 MR of CD to stearic acid-PEG-SA = 0.35
CD.siNeg.stearic acid-PEG-SA	WR of CD to siNeg = 10 MR of CD to DSPE-PEG = 0.7 MR of CD to stearic acid-PEG-SA = 0.35

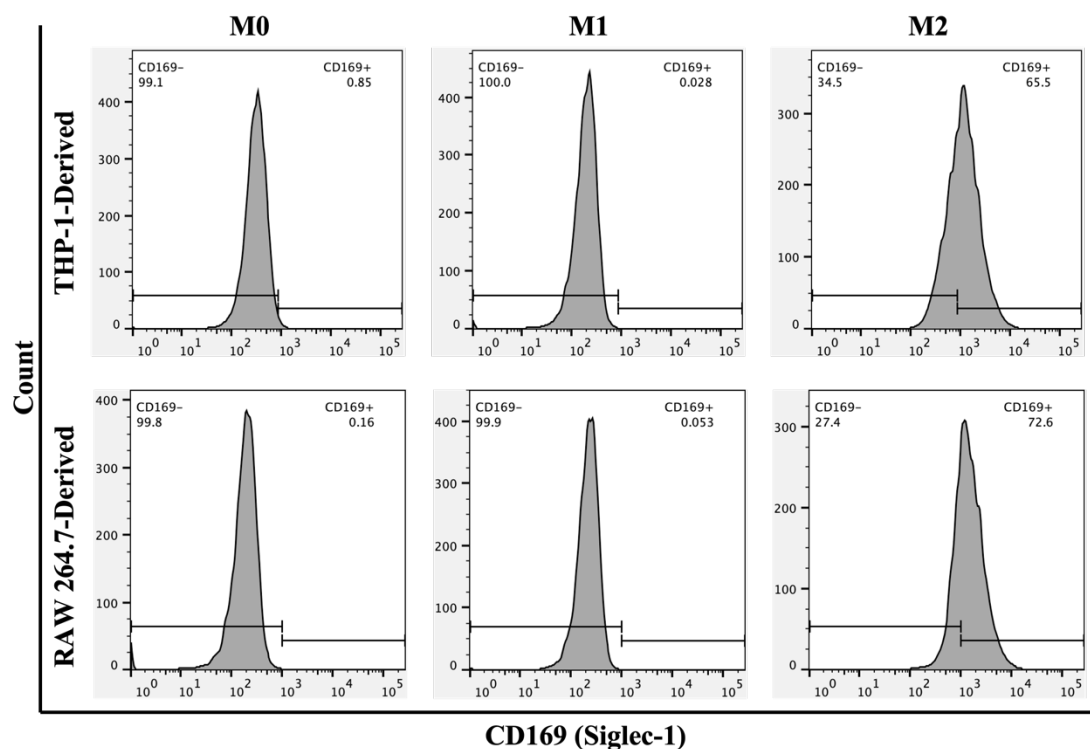


Fig. S2.2. The expression of CD169 (Siglec-1) on THP-1- and RAW 264.7-derived M0/M1/M2 macrophages.

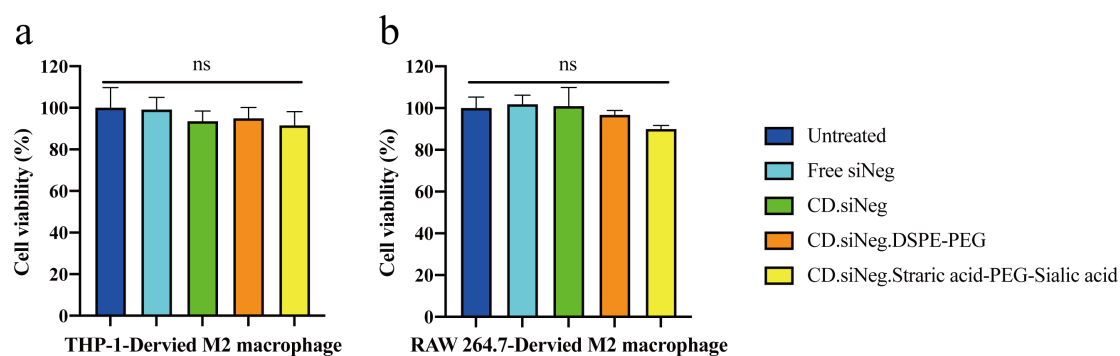


Fig. S2.3. Cytotoxicity of CD-based formulations with siNeg (100 nM) was measured by CCK-8 assay (mean $\pm$ SD, n = 6, ns = not significant).

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Chapter 3 M2pep-Modified Cyclodextrin-siRNA Nanoparticles Modulate the Immunosuppressive Tumour Microenvironment for Prostate Cancer Therapy (*in vitro* part)

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3.1 Abstract

Prostate cancer (PCa) is the most prevalent cause of cancer fatalities in men. Conventional strategies, such as surgery, radiation or chemotherapy, face challenges including poor prognosis and resistance. Therefore the development of new improved strategies is vital to enhance patient outcomes. Recently, immunotherapy has shown potential in the treatment of a range of cancers including PCa. Tumour-associated macrophages (TAMs) play an important role in the tumour microenvironment (TME) and reprogramming of TAMs is associated with remodelling the TME. Colony stimulating factor-1/colony stimulating factor-1 receptor (*CSF-1/CSF-1R*) signalling pathway is closely related to the polarization of TAMs. The downregulation of *CSF-1R*, using small interfering RNA (siRNA), has been shown to achieve the reprogramming of TAMs, from the immunosuppressive M2 phenotype to the immunostimulatory M1 one. To maximise specific cellular delivery an M2 macrophages-targeting peptide, M2pep, was formulated with an amphiphilic cationic β -Cyclodextrin (CD) incorporating *CSF-1R* siRNA. The resulting nanoparticles (NPs) increased M2 macrophage targeting both *in vitro* and *in vivo*, promoting the release of M1 factors and simultaneously downregulating the levels of M2 factors through TAM reprogramming.

3.2 Keywords

Targeted nanoparticles, Functionalised Cyclodextrin; Tumour microenvironment, M2pep; M2 Macrophages; CSF-1R; Prostate Cancer; Immunotherapy

3.3 Introduction

In 2022 in U.S.A. males approximately 268,490 new cases and about 34,500 deaths from prostate cancer (PCa), estimated to account for 27% of new cancer cases and 11% of deaths, were reported making it the most prevalent and second-deadliest cancer type.(Siegel et al., 2022) Current treatment options are limited and frequently ineffective, therefore an urgent need exists to develop more effective clinical alternatives. Recently immunotherapy has shown promise in the treatment of a range of cancers including PCa, which is considered to be an immunosuppressive cancer. TAMs have been regarded as pro-tumoural macrophages and consequently are linked to adverse prognoses in cancers, such as PCa.(Fridman et al., 2017, Yang et al., 2020) TAMs and tumour cells are the main cellular components constituting the TME, and crosstalk between these cell types is the important regulator of tumour growth and metastasis.(Yang et al., 2020) TAMs are generally classified into two phenotypes, M1 and M2.(Guerriero, 2018) M1 TAMs are regarded as an anti-tumorigenic phenotype, which release M1 factors, such as interleukin-12 (IL-12) to directly kill tumour cells and recruit cytotoxic T cells for indirect anti-tumour effects.(Veglia et al., 2018, Biswas and Mantovani, 2010) In contrast, M2 TAMs described as pro-tumoural macrophages are associated with tumour immunosuppression and angiogenesis.(Martinez et al., 2008, Porta et al., 2009) The number of M2 TAMs increases with tumour growth and gradually dominates the immunosuppressive tumour microenvironment (TME). The immunosuppressive action of M2 TAMs is due to the release of M2 factors, such as interleukin-6 (IL-6), which can cause resistance to immunotherapeutics.(Pathria et al.,

2019, Biswas and Mantovani, 2010) It is known that cytotoxic T lymphocytes (CTLs) are key factors in immunotherapy. In immunosuppressive cancers such as PCa, the TME suppresses the infiltration of CTLs by multiple mechanisms, including M2 factors released by M2 TAMs.(Verma et al., 2022) At the same time, M2 cytokines also upregulate the level of anti-inflammatory cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs).(Yang et al., 2020) In addition, subpopulation of MDSCs, M-MDSCs, are stimulated by M2 factors to differentiate into M2 macrophages.(Mantovani et al., 2017) This cycle leads to an increasing degree of immunosuppression in the TME. Consequently, modulating the polarization of TAMs from M2 to M1 phenotypes has been reported as a promising immunotherapeutic strategy for cancer. (Pathria et al., 2019, Veglia et al., 2018, Biswas and Mantovani, 2010, Guerriero, 2018)

Colony stimulating factor-1 receptor (CSF-1R) plays a critical role in the polarization of TAMs. Downregulation of CSF-1R expression, using small interfering RNA (siRNA) or inhibitors/monoclonal antibodies, has proven to reprogram M2 macrophages to the M1 phenotype.(Qian et al., 2017, Li et al., 2020) A number of clinical trials including: clinic trail Nos.: NCT02323191 (completed, applied anti-CSF-1R and anti-PD-L1 monoclonal antibodies on solid tumours), NCT02718911 (completed, applied CSF-1R monoclonal antibody on solid tumours), and NCT01346358 (completed, applied CSF-1R monoclonal antibody on solid tumours)] have investigated this approach for cancer therapy.(Gomez-Roca et al., 2022, Falchook et al., 2021, Dowlati et al., 2021) In the

trail NCT02323191, in total of 221 patients were treated by the combination of CSF-1R and PD-L1 antibodies. Tumour biopsy analyses demonstrated increased activated CD8⁺ tumour infiltrating T lymphocytes (TILs) in the combination group compared with untreated group, which suggested the great potential of CSF-1R in cancer immunotherapy.

One of the main challenges in manipulating the TME for therapeutic outcomes is the ability to target the cells of interest and avoid non-specific delivery. Cyclodextrins have been used for gene delivery and have shown transfection efficacy in a range of diseases, including cancers and central nervous system (CNS) diseases.(O'Mahony et al., 2013, Haley et al., 2020) Previously, sialic acid-targeted CD-based NPs containing siRNA have been studied in PCa immunotherapy, the results illustrated the ability of the NPs to target the macrophages and remodel the TME.(Sun et al., 2023) M2 macrophage-targeting peptide, M2pep, is an artificially designed peptide, the sequence of which was selected by Pun et al. using subtractive phage biopanning.(Cieslewicz et al., 2013) The application of M2pep as a targeting ligand has been investigated previously where polyethylenimine-stearic acid-based micelles with M2pep successfully co-delivered CSF-1R siRNA and PI3K- γ inhibitor to reprogram TAMs for pancreatic cancer therapy(Li et al., 2020). In addition, a dual targeting ligand approach including α -peptide [a scavenger receptor B type 1 (SR-B1) targeting peptide] and M2pep was utilised for the delivery of lipid nanoparticles to treat melanoma(Qian et al., 2017). In the current work, we are investigating a M2pep-targeted amphiphilic cyclodextrin

(CD)-based NP to incorporate siRNA. The ability of the resulting CD-siRNA NPs to reprogram the TAM was initially studied *in vitro* and the optimised formulation was subsequently investigated in an *in vitro* co-culture model of prostate cancer cells and macrophages.

3.4 Materials and Methods

3.4.1 Materials

1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol)-2000 (DSPE-PEG) and DSPE-PEG-maleimide (DSPE-PEG2000-Mal) were purchased from Nanocs Inc. (New York, NY, USA). M2pep (sequence: N'-YEQDPWGVKWWY-C') was synthesized by TAG Copenhagen A/S (Frederiksberg, Denmark). Human GAPDH siRNA (sense sequence: 5'-GUGGAUAUUGUUGCCAUCAtt-3'), mouse GAPDH siRNA (sense sequence: 5'-GGCCGAGAAUGGGAAGCUUtt-3'), human CSF-1R siRNA (sense sequence: 5'-GUUGAGACCUUAGAGCACAtt-3'), and mouse CSF-1R siRNA (sense sequence: 5'-GGUCUUACGCAAACGGUCtt-3') were purchased from Thermo Fisher Scientific (Waltham, MA USA). Negative control siRNA (siNeg), 6-FAM-labeled negative control siRNA (FAM-siNeg), and Cyanine 5-labeled negative control siRNA (Cy5-siNeg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNA extraction kits and RNA reverse transcription kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). All primers, RT-PCR kits, and immunofluorescence antibodies were purchased from Thermo Fisher Scientific (Waltham, MA USA). Flow cytometry antibodies and ELISA kits were purchased from BioLegend (San Diego, CA,

USA).

3.4.2 Cells

The Homo sapiens monocyte cell line THP-1 was donated by Cancer Research @UCC, University College Cork, Ireland. The mouse macrophage cell line RAW 264.7, the Homo sapiens prostatic adenocarcinoma bone metastasis cell line PC-3, and the mouse prostatic adenocarcinoma cell line TRAMP-C1 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

THP-1 cells were cultured in RPMI-1640 Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), D-(+)-Glucose (Sigma-Aldrich), 0.05 mM beta-mercaptoethanol (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich). RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS and 1% penicillin-streptomycin. PC-3 cells were cultured in RPMI-1640 Medium (Sigma-Aldrich) supplemented with 10% FBS and 1% penicillin-streptomycin. TRAMP-C1 cells were cultured in DMEM supplemented with 5% Nu Serum IV (Corning), 5% FBS, 5 µg/mL insulin (Sigma-Aldrich), 10 nM 5 α -Androstan-17 β -ol-3-one (Sigma-Aldrich) and 1% penicillin-streptomycin.(Guo et al., 2012a, Guo et al., 2012b) All cells were incubated at 37 °C supplied with 5% carbon dioxide.

The monocyte cell line, THP-1, was differentiated to macrophages by treating (48 hours) with 200 nM 12-O-Tetradecanoylphorbol 13-acetate (PMA, Sigma-Aldrich).(Pantazi et al., 2021, Zheng et al., 2018) Subsequently, 20 ng/mL lipopolysaccharides (LPS, Sigma-Aldrich) or 20 ng/mL recombinant human interleukin-4 (IL-4, PeproTech) was used to differentiate THP-1-derived macrophages to M1 or M2 phenotypes.(Faass et al., 2021, Liu et al., 2021, Ma et al., 2020)

For RAW 264.7 cells, 20 ng/mL LPS or 20 ng/mL of murine IL-4 was used to promote differentiation from the M0 phenotype to M1 or M2 macrophages.(Chen et al., 2020, Sun et al., 2020, Song et al., 2018)

3.4.3 Synthesis of DSPE-PEG-M2pep

M2pep and DSPE-PEG-Mal were conjugated via the Michael addition; reaction of the cystine thiol group on M2pep with the DSPE-PEG-Mal maleimide group.(Li et al., 2020) Briefly, DSPE-PEG-Mal (11.70 mg, 0.00585 mmol, 1 Equiv) and M2pep (15.44 mg, 0.00877 mmol, 1.5 Equiv) were suspended in chloroform/methanol (3 mL, 2/1, v/v). The mixture was sonicated for 30 mins to promote dissolution. Then triethylamine (20 μ L) was added as a catalyst. The solution was stirred under a nitrogen atmosphere for 2 days at room temperature. Thin layer chromatography (TLC, silica: chloroform/methanol) comparison of a reaction sample and starting materials, observed under a UV lamp, indicated the complete consumption of DSPE-PEG-Mal. The reaction solution was dialyzed against deionized water (DIW) in a 3.5k Mw cut-off

dialysis cassette (Thermo Fisher) for 48 h with outer water replacement every 12 h. The product, DSPE-PEG-M2pep (11.29mg, 0.00300 mmol, 51.33 mol% based on DSPE-PEG-Mal) as a tan coloured solid, was obtained by freeze-drying and stored at -20°C. DSPE-PEG-M2pep was identified by NMR (Bruker Avance 600 UltraShield NMR spectrometer, USA) and Fourier Transform Infra-Red (FT-IR, Perkin Elmer Spectrum Two, Attenuated Total Reflectance) spectrometry.

3.4.4 Nanoparticle preparation

The amphiphilic cationic β -cyclodextrin (CD) was prepared as previously reported.(Guo et al., 2017, O'Mahony et al., 2012) The CD was dissolved in water and sonicated at 60 °C for 1 h to promote dissolution. For the basic nanoparticles, CD.siRNA, the CD and siRNA aqueous solutions were incubated at 25 °C for 0.5 h at 450 rpm in a ThermoMixer (Eppendorf). The non-targeted and targeted formulations CD.siRNA.DSPE-PEG and CD.siRNA.DSPE-PEG-M2pep were achieved by a “post-insertion” method,(Guo et al., 2017) where CD.siRNA complexes were incubated with DSPE-PEG or DSPE-PEG-M2pep at 60 °C for 1 h, at 450 rpm in a ThermoMixer (Eppendorf, Hamburg, Germany).

3.4.5 Physicochemical characterisation of the nanoparticles

To optimize the optimum ratio of CD and siRNA, CD.siRNA NPs at different weight ratios (WR) were examined by agarose gel electrophoresis to monitor the binding of siRNA. Free siNeg or CD.siNeg NPs ranging from WR 5:1 to 20:1 were loaded on a

1% (w/v) agarose gel in Tris/Borate/EDTA (TBE) buffer containing a nucleic acid stain (NBS Biologicals Ltd). The weight of siNeg was fixed as 0.5 µg. Electrophoresis was performed using 120 V for 0.5 h, results were imaged and photographed under UV.

Nanoparticle sizes and surface charges were measured by dynamic light scattering (DLS, Malvern Nano-ZS, Malvern Instruments). CD.siRNA complexes were co-formulated with DSPE-PEG in the range of 0.14:1 to 7:1 MR to optimize the ratio of CD and DSPE-PEG. After identification of the optimum WR of CD to siRNA and MR of CD to DSPE-PEG, three formulations of CD.siRNA, CD.siRNA.DSPE-PEG, and CD.siRNA.DSPE-PEG-M2pep were examined by agarose gel electrophoresis and DLS.

The morphology of the different formulations was visualised by transmission electron microscopy (TEM, JEOL 2000 FXII, Jeol Ltd). CD.siNeg NPs were placed on copper grids for 3 mins, stained by uranyl acetate and imaged after 12 h.

The stability of siRNA in NPs following incubation in 50% FBS for 4 or 24 h was assessed, where the heparin released siRNA was analyzed on an agarose gel. The NPs were incubated with salt-containing media (90% Opti-MEM media, Thermo Fisher) for up to 3 days and sizes were measured by DLS every 24 h.

3.4.6 In vitro cellular uptake in M2 macrophages

THP-1 and RAW 264.7 cells were differentiated to M2 macrophages as described above,

the cellular uptake of targeted and non-targeted NPs containing FAM-siNeg were monitored by flow cytometry and fluorescence microscopy. 1.0×10^5 M2 Macrophages were seeded in 24-well plates, and either targeted or non-targeted NPs were added onto the cells for 6 or 24 h, post-transfected cells were analyzed by flow cytometry or stained by a nuclear acid dye for fluorescence microscopy.

To confirm the targeting effects of NPs, a competitive uptake study was performed, where free M2pep was added to the cells for 1 h, subsequently the cells were incubated with either targeted or non-targeted NPs for 6 h. After incubation, the cells were analysed by flow cytometry.

To verify the specificity of the ligand, GAPDH gene knockdown by either targeted or non-targeted NPs containing GAPDH siRNA in both THP-1- and RAW 264.7-derived M0/M1/M2 macrophages was quantified. All macrophage phenotypes were differentiated as described above, and seeded on 24-well plates, cells were incubated with the CD formulations for 6 h, the media was then replaced by fresh media for a further 42 h. Post-incubation, cells were collected and the expression of GAPDH was measured by RT-qPCR, beta-actin was used as the internal reference.

3.4.7 In vitro cytotoxicity of nanoparticles

To examine the cytotoxicity of the three CD formulations, 1.0×10^4 M2 macrophages were seed in a 96-well plate, and all 3 CD NPs were added to cells for 2 days, the Cell

courting kit - 8 (CCK-8) solution was added and the viability determined using a plate reader (Spark, Tecan, Switzerland) at 460 nm. The cell viability was calculated using the following equation where the untreated group was the negative control, and regarded as 100% cell viability.

$$\text{Cell Viability (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{negative control}} - \text{OD}_{\text{blank}}} \times 100 \%$$

3.4.8 In vitro downregulation of CSF-1R

THP-1- and RAW 264.7-derived M2 macrophages were seeded in 24-well plates, different formulations, including Free siRNA, CD.siRNA, CD.siRNA.DSPE-PEG, CD.siRNA.DSPE-PEG-M2pep containing CSF-1R siRNA, and CD.siRNA.DSPE-PEG-M2pep containing siNeg were incubated with the cells for 6 h, the media was then replaced by complete media for a further 42 h. The expression of CSF-1R was analysed by RT-qPCR, using beta-actin as the internal reference.

3.4.9 In vitro reprogramming of TAMs and remodelling of the TME

To examine the reprogramming of the M2 macrophages, post-transfected cells were stained using the appropriate antibodies, human (CD68) or mouse (F4/80), and the relevant M1 (CD86) or M2 (CD206) macrophages markers were measured by flow cytometry.

In addition, to further explore the influence of reprogramming of TAMs on

immunotherapy, a Transwell model was established to simulate the tumour microenvironment by co-culturing macrophages and prostate cancer cells. M2 macrophages were seeded into the upper chamber of the Transwell model, and PCa cells were seeded on the lower well. THP-1-derived M2 macrophages were co-cultured with PC-3 cells, and RAW 264.7-derived M2 macrophages were co-cultured with TRAMP C1 cells. Different formulations were added into upper chambers for 48 h, post-transfection the media from the lower wells were collected and M1/M2 cytokines IL-12/IL-6 protein content was measured by ELISA. Total RNA from the upper chambers was extracted to measure M1 (IL-12, CXCL-10, TNF- α) and M2 (IL-6, IL-10, Arg-1, CD163) factor expression by RT-qPCR. The apoptosis of cancer cells was analyzed by propidium iodide (PI)/Annexin V double staining and measured by flow cytometry.

3.4.10 Statistical analysis

The statistical analysis was performed with Prism 8.0 (GraphPad, San Diego, CA, USA). Data were shown as mean $\pm$ standard deviation (mean $\pm$ SD) from at least 3 independent experiments. One-way analysis of variance (one-way ANOVA), followed by Dunnett or Tukey post hoc tests and Student's t-test was used to calculate the statistical significance between three or more groups and two groups. A p-value < 0.05 was considered a significant difference.

3.5 Results and Discussion

3.5.1 Preparation and in vitro characterization of the nanoparticles

The M2pep targeting moiety was successfully conjugated to DSPE-PEG via the Michael Reaction. (Li et al., 2020) The structure of the product DSPE-PEG-M2pep was confirmed by $^1\text{H}/^{13}\text{C}$ NMR and FT-IR (Fig. S3.1).

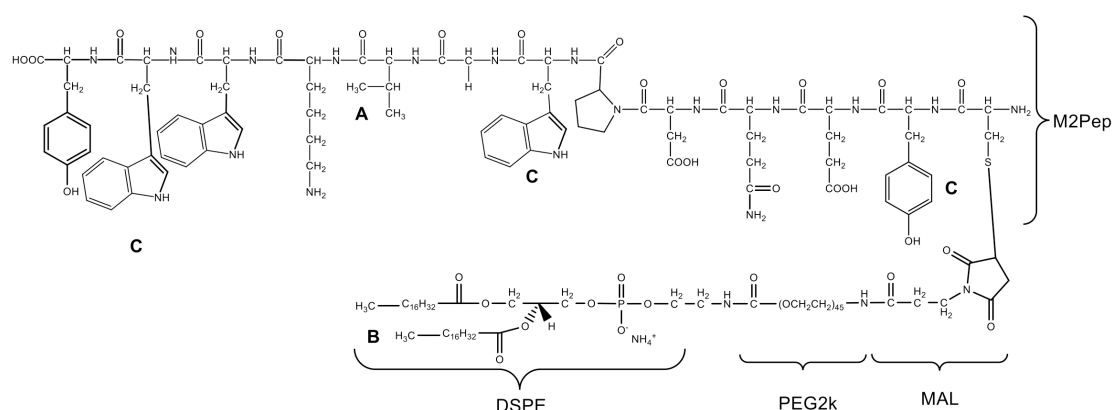


Fig. 3.1. The chemical structure of the ligand, DSPE-PEG-M2pep.

Key signals associated with M2pep are evident in the proton spectrum: tryptophan and tyrosine aromatic residues (marker C in Fig. 3.1 and S3.1) in the 6.6 - 8.5 ppm region. Methyl group protons associated with valine and also terminal methyl groups of DSPE alkyl chains (marker A and B respectively in Fig. 3.1 and S3.1) are observed at 0.7 - 0.9 ppm. A strong PEG2k signal at 3.3 - 3.5 ppm and C17 alkyl chain protons resonating in the 1.0 - 1.9 ppm area are diagnostic for DSPE-PEG. The carbon spectrum has corresponding aromatic signals in the 110 - 130 ppm region (tryptophan and tyrosine), PEG2k carbons (-OCH₂-) at 70 ppm and alkyl chain carbons in the range 20 - 35 ppm.

The FT-IR spectrum of the conjugate is shown in Fig. S3.1 in comparison with M2pep and DSPE-PEG-Mal starting materials. Diagnostic signals observed for the conjugate are amide I (1633cm^{-1}) and amide II (1547cm^{-1}), both associated with M2pep; along with PEG (1094cm^{-1}).

^1H NMR (DMSO, ppm): δ 0.7 – 0.9 (m, 12H), 1.1 - 1.9 (m, 24H), 2.0 – 2.1 (m, 4H), 2.2 – 2.3 (m, 3H), 2.6– 3.9 (m, 192H), 4.05 (s, 1H), 4.1 – 4.6 (m, 10H), 4.82 (s, 1H), 4.98 (s, 1H), 5.10 (s, 1H), 6.60 (m, 4H), 6.75 (s, 1H), 6.9 – 7.4 (m, 15H), 7.50 (m, 2H), 7.60 (m, 1H), 7.7 – 8.5 (m, 11H), 9.20 (br. s, 1H), 10.8 (m, 2H).

^{13}C NMR (DMSO, ppm): δ 19.63, 22.45, 26.84, 27.35, 28.14, 28.63, 29.00, 29.28, 29.84, 30.08, 31.80, 35.85, 36.06, 36.57, 39.82, 39.88, 39.96, 40.03, 40.06, 40.09, 40.20, 40.23, 40.31, 40.34, 40.48, 40.86, 44.09, 46.15, 46.72, 47.31, 52.49, 53.87, 54.56, 60.66, 63.51, 69.55, 70.02, 70.15, 70.18, 70.25, 72.79, 73.11, 74.48, 75.92, 102.22, 111.70, 115.40, 118.67, 118.76, 121.25, 121.34, 124.01, 124.09, 130.58.

FT-IR cm^{-1} : 3385 (O-H), 2956, 2920, 2850 (C-H), 1633 (amide I), 1547 (amide II), 1094 (C-O-C).

Initially weight ratios (WR) of CD to siRNA in a range of WR 5: 1 to WR 20: 1 were formulated, in all cases full binding (agarose gel electrophoresis) of siRNA was achieved (Fig. S3.2A). Particle size and surface charge were measured by DLS (Fig. S3.2B). Optimum results were detected as follow with WR 10: 1, CD to siRNA: size 171.1 ± 20.6 nm, charge 13.2 ± 2.6 mV, and PDI of 0.23. Therefore, CD.siRNA WR 10:

1 was used for all further studies (Table 3.1).

The CD.siRNA NPs were PEGylated by the insertion of DSPE-PEG.(Guo et al., 2017, Gao et al., 2012) Optimisation studies identified the following CD.siRNA.DSPE-PEG (WR of CD to siRNA = 10, MR of CD to DSPE-PEG = 0.35:1) (Fig. S3.3): size 189.6 ± 29.1 nm, charge 14.2 ± 2.7 mV, and PDI of 0.35.

Following incorporation of the targeting moiety, DSPE-PEG-M2pep, full binding of siRNA was maintained (Fig. 3.2A). The targeted NPs, CD.siRNA.DSPE-PEG-M2pep, had a size of 252.5 ± 16.5 nm and a charge of 10.8 ± 2.5 mV (Fig. 3.2B and Table 3.1). The increasing size compared with the non-targeted NPs suggests the successful incorporation of M2pep ($n = 6$, $**p < 0.01$).

The morphology of the NPs was imaged by TEM (Fig. 3.2C). All particles are spherical, the presence of PEG appears to give a slight halo effect, sizes were consistent with the DLS results and our previously published CD NPs data.(Guo et al., 2017, Evans et al., 2016)

The stability of all three formulations was assessed following incubation with 50% serum. The heparin release data indicate siRNA is stable following formulation into the NPs (Fig. S3.4A). In addition, relative to naked siRNA the targeted NPs maintained siRNA stability following incubation in serum for up to 4 and 24 hours (Fig. S3.4B).

These results were consistent with previous stability reports for similar CD NPs obtained following exposure to human blood.(Guo et al., 2017) In addition, stability in 90% Opti-MEM medium (salt-containing) for 3 days was enhanced by the presence of PEG in the targeted and PEGylated non-targeted NPs thus implying the potential for longer circulation *in vivo* (Fig. S3.4C).

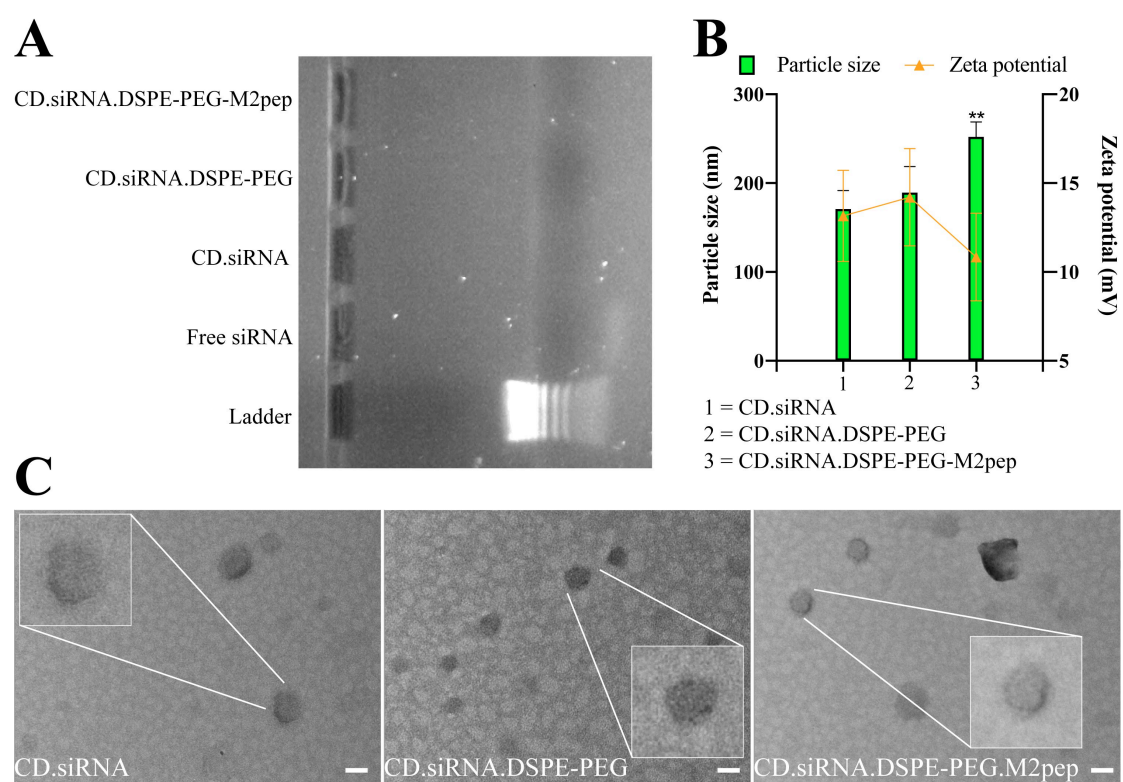


Fig. 3.2. (A) CD formulations contained 0.5 μg siRNA was confirmed by 1% agarose gel electrophoresis and photographed by UV. (B) Particle sizes and zeta potentials of CD NPs ($n = 6$, mean $\pm$ SD, $**p < 0.01$ compared with CD.siRNA.DSPE-PEG NPs). (D) Morphology of CD formulations (scale bar = 200 nm).

Table 3.1. Size and charge of the optimised CD Formulations

Formulation	Size (nm)	PDI	Charge (mV)
CD.siRNA	171.1 ± 20.6	0.23	13.2 ± 2.6
CD.siRNA.DSPE-PEG	189.6 ± 29.1	0.35	14.2 ± 2.7
CD.siRNA.DSPE-PEG-M2pep	252.5 ± 16.5	0.25	10.8 ± 2.5

PDI = polydispersity index; mean ± SD, n = 6.

3.5.2 Cellular uptake into M2 macrophages

THP-1 and RAW 264.7 were differentiated into M2 macrophages as described above, identified by flow cytometry to measure the macrophage markers (Fig. S3.5), and used to investigate cellular uptake. In THP-1-derived M2 macrophages, uptake was higher with the targeted NPs versus the non-targeted (Fig. S3.6A & Fig. 3.3A, 19.49 ± 0.29% FAM+ vs 5.22 ± 0.31% FAM+ after 6 h, and 43.10 ± 1.45% FAM+ vs 11.97 ± 0.60% FAM+ after 24 h, n = 3, ** $p < 0.01$). Similar results were observed in RAW 264.7-derived M2 macrophages (Fig. S3.6B & Fig. 3.3B), 52.00 ± 1.81% FAM+ vs 8.46 ± 0.19% FAM+ after 6 h, and 78.40 ± 1.45% FAM+ vs 12.67 ± 0.43% FAM+ after 24 h (n = 3, ** $p < 0.01$). These results indicate that the incorporation of the targeting ligand, M2pep, enhanced the uptake of the NPs. The higher uptake of the targeted NPs after 6 and 24 h was confirmed by fluorescent microscopy (Fig. 3.3C & D). It has been reported that M2pep-targeting formulations increased M2 macrophage cellular internalization by 4.2-fold versus non-targeted NPs.(Li et al., 2020) In this study, a similar increase was observed, after the longer transfection time (24 h), the M2pep-targeting NPs enhanced the levels of cellular internalization by human and mouse M2

macrophages up to 3.6- and 6.2-fold respectively compared with the non-targeted CD.siRNA.DSPE-PEG NPs.

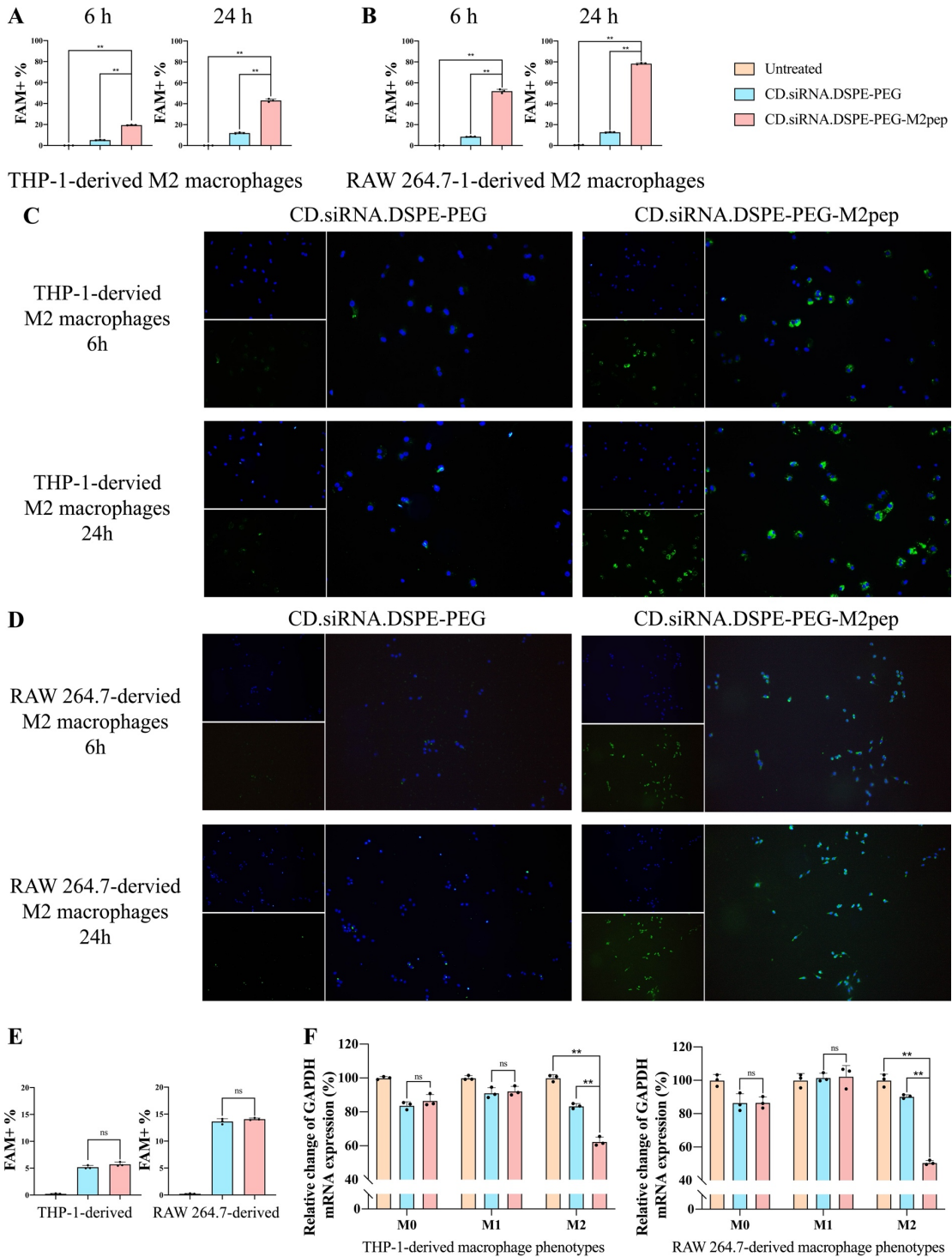


Fig 3.3. Cellular uptake of CD.siRNA.DSPE-PEG or CD.siRNA.DSPE-PEG-M2pep

formulations containing FAM-siRNA in (A) THP-1- or (B) RAW 264.7-derived M2 macrophages after 6 or 24 h transfection ($n = 3$, mean $\pm$ SD, $**p < 0.01$). (C - D) Cellular uptake imaged by fluorescence microscopy (blue: DAPI, green: FAM-siRNA). (E) Competitive uptake, free M2pep was added to THP-1- or RAW 264.7-derived M2 macrophages prior to the addition of the formulations. The CD.siRNA.DSPE-PEG or CD.siRNA.DSPE-PEG-M2pep nanoparticles containing FAM-siRNA were incubated with the cells for 6 h ($n = 3$, ns = not significant). (F) M2 macrophages-targeting efficacy (gene silencing) using CD formulations with GAPDH siRNA in THP-1- or RAW 264.7-derived macrophages, cells were incubated with CD complexes for 6 h followed by replacement with complete media for a further 44 h incubation, analysis by RT-qPCR ($n = 4$, ns = not significant, mean $\pm$ SD, $**p < 0.01$).

To confirm the higher uptake was due to the targeting ligand, a competitive uptake study was performed. Following exposure of the cells to free M2pep for 1 h, subsequently no difference in uptake of the targeted versus the non-targeted NPs was detected ($p = 0.1447$ in THP-1-derived M2 macrophages; $p = 0.2898$ in RAW 264.7-derived M2 macrophages) (Fig. S3.6C & Fig. 3.3E). These results indicate the key role of M2pep in the enhanced cellular uptake of the targeted NP.

To verify the gene silencing efficacy of the targeted NPs, GAPDH siRNA was used. Post transfection (6 h) no gene knockdown was detected in M0 or M1 macrophages, in contrast in the M2 macrophages, expression of GAPDH mRNA was significantly

decreased by the targeted versus the non-targeted NPs (Fig. 3.3F, $62.45 \pm 2.68\%$ vs $83.42 \pm 1.52\%$ in THP-1-derived M2 macrophages, and $50.62 \pm 1.35\%$ vs $90.26 \pm 1.09\%$ in RAW 264.7-derived M2 macrophages, $n = 3$, $**p < 0.01$). These results indicate that M2pep labelled NPs are capable of cell specific delivery mainly to M2 macrophages. Compared with sialic acid-targeted CD-based NPs, the M2pep-targeting ligand produced a higher cellular uptake in M2 macrophages (1.15- and 1.23-fold higher in THP-1- and RAW 264.7-derived M2 macrophages after 24 h transfection), indicating the stronger M2 macrophages targeting ability of M2pep.(Sun et al., 2023)

3.5.3 In vitro macrophage reprogramming

Initially the cytotoxicity of the CD formulations was assessed in both THP-1 and RAW 264.7-derived macrophages using a CCK-8 kit, results show no significant toxicity ($n = 3$, $p > 0.05$), under the conditions studied (Fig. S3.7). While the positive control group, RNAiMAX presented significant cytotoxicity on both THP-1- and RAW 264.7-derived M2 macrophages ($n = 3$, $**p < 0.01$).

The ability of the CD NPs to silence CSF-1R was assessed in both THP-1- or RAW 264.7-derived M2 macrophages (Fig. 3.4A). The targeted NPs significantly downregulate ($n = 4$, $**p < 0.01$) the expression levels of CSF-1R mRNA in both human (42.18 % knockdown) and mouse M2 macrophages (57.65% knockdown). The decrease in gene expression, relative to controls, detected with the PEGylated non-targeted nanoparticles ($n = 4$, $**p < 0.01$) may be indicative of enhanced stability due

to the presence of PEG. In addition, compared with the positive control group, RNAiMAX.siRNA, the targeted NPs presented similar CSF-1R mRNA downregulation in RAW 264.7-derived M2 macrophages ($p = 0.0968$). As previously reported, NPs containing CSF-1R siRNA modified with M2pep-targeting ligand downregulated up to 85% expression of CSF-1R in pancreatic cancer therapy *in vitro*. (Li et al., 2020) In addition, a dual-targeting ligand [M2pep and α -peptide (an SR-B1 targeting peptide)] for treatment of melanoma resulted in 81% gene knockdown *in vitro*. (Qian et al., 2017)

To verify that downregulation of CSF-1R achieved the reprogramming of TAMs from the M2 to the M1 phenotype, specific macrophage markers were measured in the post-transfected cells (Fig. S3.8A & B). Relative to untreated controls, in the THP-1 derived macrophages, the targeted NPs significantly increased the M1 macrophages (CD68+/CD86+) to $72.97 \pm 0.21\%$ and decrease the M2 macrophages (CD68+/CD206+) to $25.03 \pm 1.10\%$ (Fig. 3.4B & C, $n = 3$, $**p < 0.01$). Similar results were observed in mouse macrophages (Fig. S3.8C & D), where the M1 macrophages (F4/80+/CD86+) were increased to $71.90 \pm 1.57\%$ and the M2 macrophages (F4/80+/CD206+) were decreased to $26.37 \pm 1.21\%$ following exposure to the targeted NPs (Fig. 3.4D & E, $n = 3$, $**p < 0.01$). The data show that downregulation of CSF-1R resulted in an increased expression of the M1 markers indicating the successful reprogramming of the TAM to the M1 phenotype.

Compared with sialic acid-targeted NPs containing CSF-1R siRNA, the M2pep-based formulation presented higher CSF-1R gene silencing efficiencies (increased by 1.43- and 1.48-fold in THP-1- and RAW 264.7-derived M2 macrophages, respectively) and TAMs reprogramming rates (increased by 1.35- and 1.07-fold in THP-1- and RAW 264.7-derived M2 macrophages, respectively), the higher transfection efficiencies are mainly due to the higher cellular uptake mediated via M2pep.(Sun et al., 2023)

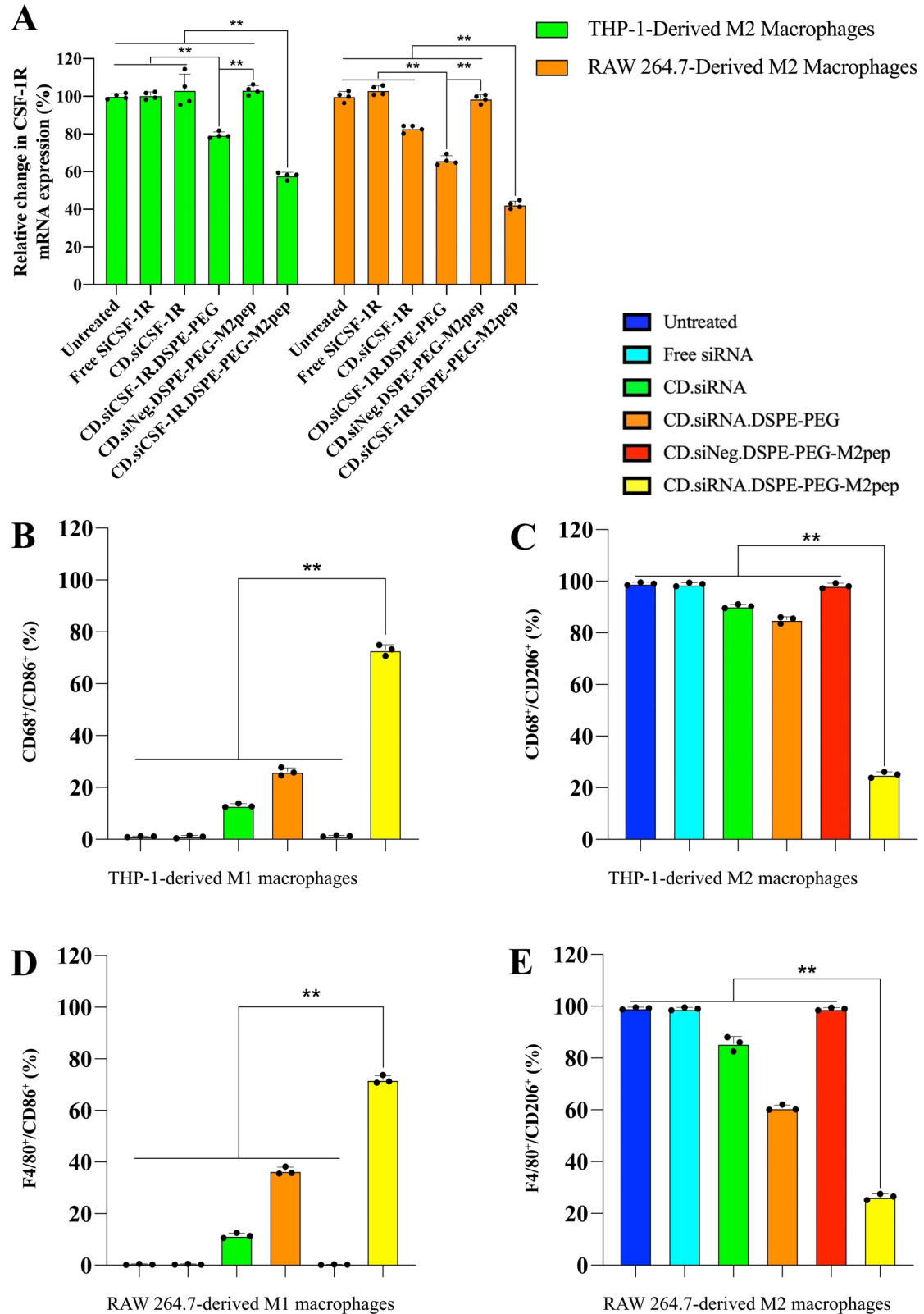


Fig. 3.4. (A) *In vitro* downregulation of CSF-1R mRNA, formulations were incubated with THP-1- or RAW 264.7-derived M2 macrophages for 6 h followed by replacement with complete media for a further 44 h incubation (n = 4, ns = not significant, * $p < 0.05$,

$**p < 0.01$). (B-E) Percentage of human/mouse macrophages markers CD68/F4/80 and M1/M2 markers CD86/CD206 expression in THP-1- or RAW 264.7-derived macrophages after transfection by different formulations ($n = 3$, mean $\pm$ SD, $**p < 0.01$).

3.5.4 *In vitro anti-tumour effects*

The macrophage reprogramming for killing tumour cells was monitored using a Transwell co-culture model where THP-1- or RAW 264.7-derived M2 macrophages were seeded into the upper chamber and human prostate cancer PC-3 cells or mouse prostate cancer TRAMP-C1 cells were seeded into the lower chamber.

Following exposure to the NPs expression of the M1/M2 factors in the macrophages was measured using RT-qPCR (Fig. 3.5A - N) and expression of M1/M2 cytokines in the supernatant of the lower chamber was quantified by ELISA (Fig. 3.5O - R). Results show that the targeted NPs significantly increased the expression of M1 factors, including IL-12, CXCL10 and TNF- α , and decrease the expression of M2 factors, including IL-6, IL-10, Arg-1, and CD163 in both human and mouse macrophages ($n = 3$, $*p < 0.05$, $**p < 0.01$). The ELISA results (Fig. 3.5O - R) confirmed the upregulation of IL-12 and the downregulation of IL-6 ($n = 3$, $*p < 0.05$, $**p < 0.01$).

Apoptosis of cancer cells was measured by PI/Annexin V double staining and flow cytometry. Flow cytometry results were shown in Fig. S3.8A & B, and the percentage

of apoptosis was shown in Fig. 3.5S & T. Compared with the other groups, the targeted nanoparticles increased cancer cells apoptosis in both PC-3 cells ($31.37 \pm 0.83\%$) and TRAMP-C1 ($43.55 \pm 1.01\%$) ($n = 3$, $**p < 0.01$). The increase in apoptosis, relative to controls, detected with the non-targeted nanoparticles ($14.61 \pm 0.63\%$ in PC-3 cells; and $23.36 \pm 0.65\%$ in TRAMP-C1, $n = 3$, $**p < 0.01$) may be indicative of enhanced stability and subsequently enhanced transfection due to the presence of PEG. It has been previously reported that polyethylenimine-stearic acid-based NPs containing CSF-1R siRNA and the M2pep-targeting ligand achieved approximately 40% apoptosis of cancer cells in a Transwell co-culture model of M2 macrophages and pancreatic cancer Pan-02 cell lines.(Li et al., 2020)

In summary, the *in vitro* results indicate that NPs containing siRNA delivered using the CD-based biomaterial successfully reprogramed the TME promoting an immunostimulatory response and the degree of apoptosis reported here for prostate cancer compares well with the previously reported efficacy data in pancreatic cancer.(Li et al., 2020)

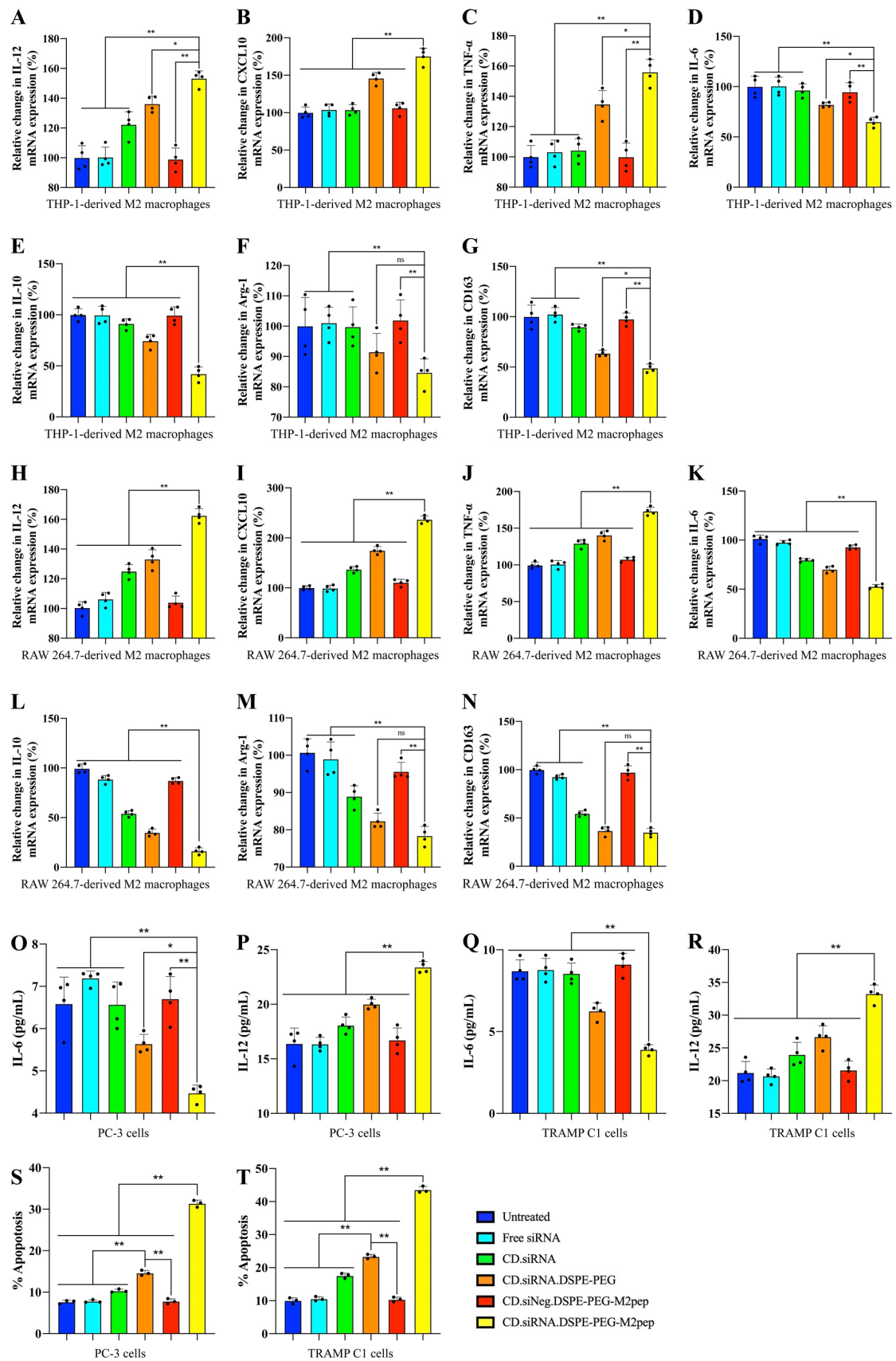


Fig. 3.5. Co-culture of M2 macrophages and prostate cancer cells. M2 macrophages were seeded in the upper chambers of the Transwell model, prostate cancer cells were

seeded in the lower chambers. RT-qPCR results of M1 cytokines in THP-1-derived macrophages: (A) IL-12, (B) CXCL-10, and (C) TNF- α , M2 cytokines in THP-1-derived macrophages: (D) IL-6, (E) IL-10, (F) Arg-1, and (G) CD163. M1 cytokines in RAW 264.7-derived macrophages: (H) IL-12, (I) CXCL-10, and (J) TNF- α , and M2 cytokines in RAW 264.7-derived macrophages: (K) IL-6, (L) IL-10, (M) Arg-1, and (N) CD163. Elisa results of cytokines in PC-3 cells: (O) IL-6, and (P) IL-12, and in TRAMP C1 cells: (Q) IL-6, and (R) IL-12 (n = 4, ns = not significant, mean $\pm$ SD, * p < 0.05, ** p < 0.01). Percentage of apoptosis of (S) PC-3 and (T) TRAMP-C1 cells in the lower chambers of the Transwell model (n = 3, mean $\pm$ SD, ** p < 0.01).

3.6 Conclusion

In the current study, a smart drug delivery system was constructed to deliver CSF-1R siRNA, the system was based on an amphiphilic cationic CD and an M2 macrophages-targeting ligand DSPE-PEG-M2pep, incorporated into the delivery system via a “post-insertion” method. The specific targeting efficacy for M2 macrophages was verified *in vitro*. In addition, the NPs reprogrammed M2 macrophages through silencing CSF-1R mRNA expression, increased M1 factor levels, and decreased M2 factors levels, and then caused PCa cells apoptosis in an *in vitro* co-culture model of PCa cells and macrophages.

3.7 CRediT authorship contribution statement

Yao Sun: Methodology, Formal analysis, Writing – original draft. Michael F. Cronin:

Methodology, Formal analysis, Writing – review & editing. Monique C.P. Mendonça: Resources, Writing – review & editing. Jianfeng Guo: Conceptualization, Supervision, Writing – review & editing. Caitriona M. O’Driscoll: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

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3.9 Supporting Information

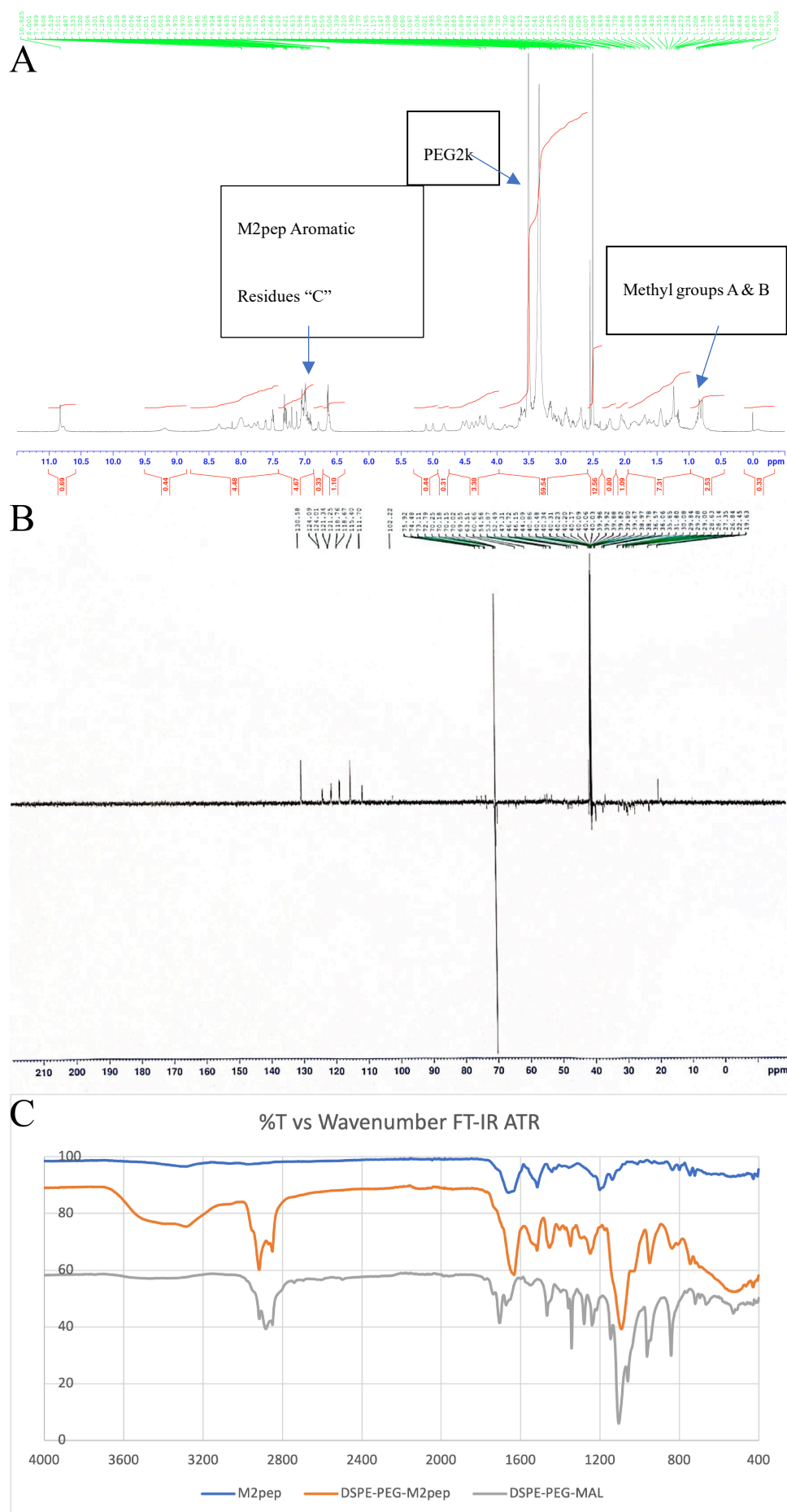


Fig. S3.1. (A) ^1H NMR spectrum of DSPE-PEG-M2pep; (B) ^{13}C NMR spectrum of DSPE-PEG-M2pep, and (C) FT-IR spectra of M2pep, DSPE-PEG-Mal, and DSPE-PEG-M2pep.

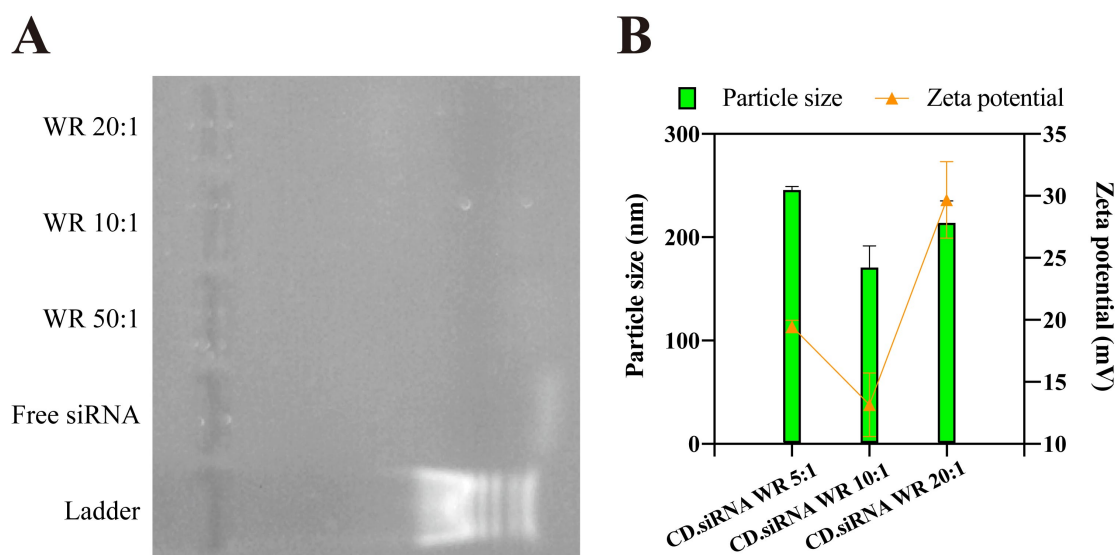


Fig. S3.2. Physicochemical characterization of CD.siRNA formulation at weight ratios ranging of CD to siRNA from 5:1 to 20:1. (a) The complexation of siRNA (0.5 μg) with CD was confirmed by using 1% agarose gel electrophoresis, the resulting gels were imaged under UV. (b) Particle sizes and zeta potentials of CD.siRNA nanoparticles ($n = 6$, mean $\pm$ SD) were measured by DLS.

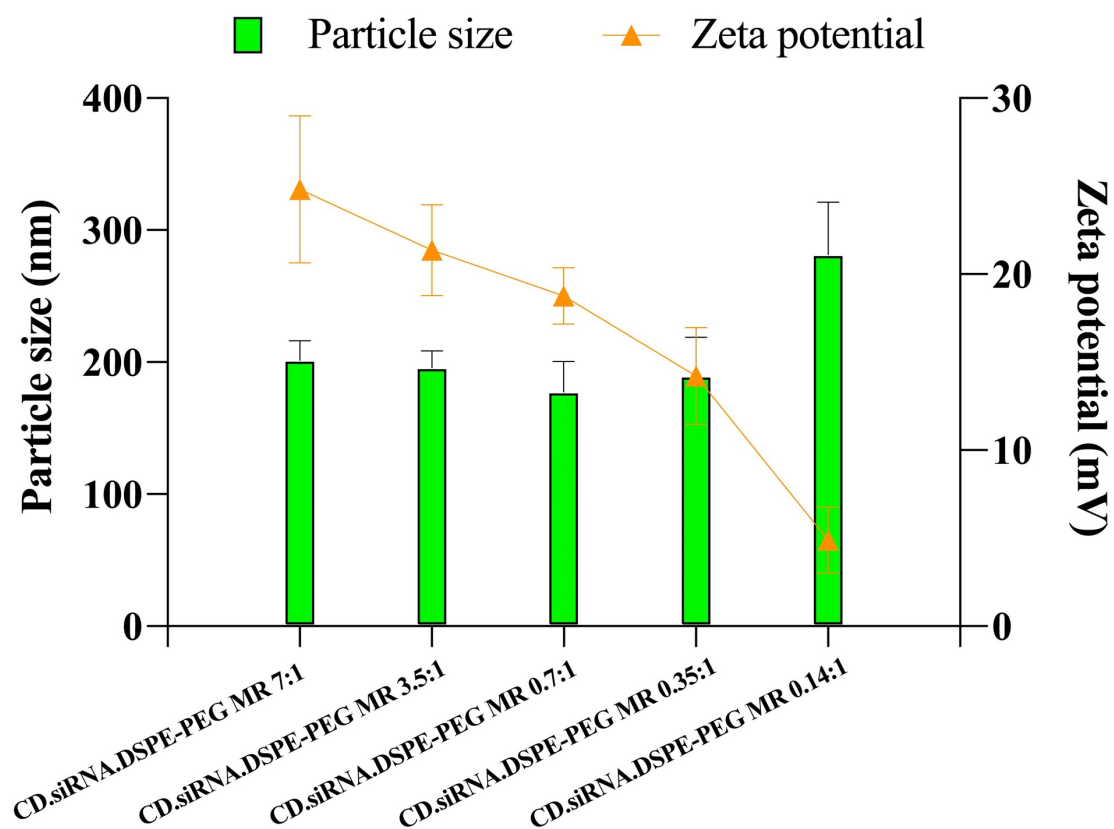


Fig. S3.3. Particle sizes and zeta potentials of CD.siRNA.DSPE-PEG nanoparticles ($n = 6$, mean $\pm$ SD) at molar ratios ranging of CD to DSPE-PEG from 0.14:1 to 7:1 were measured by DLS.

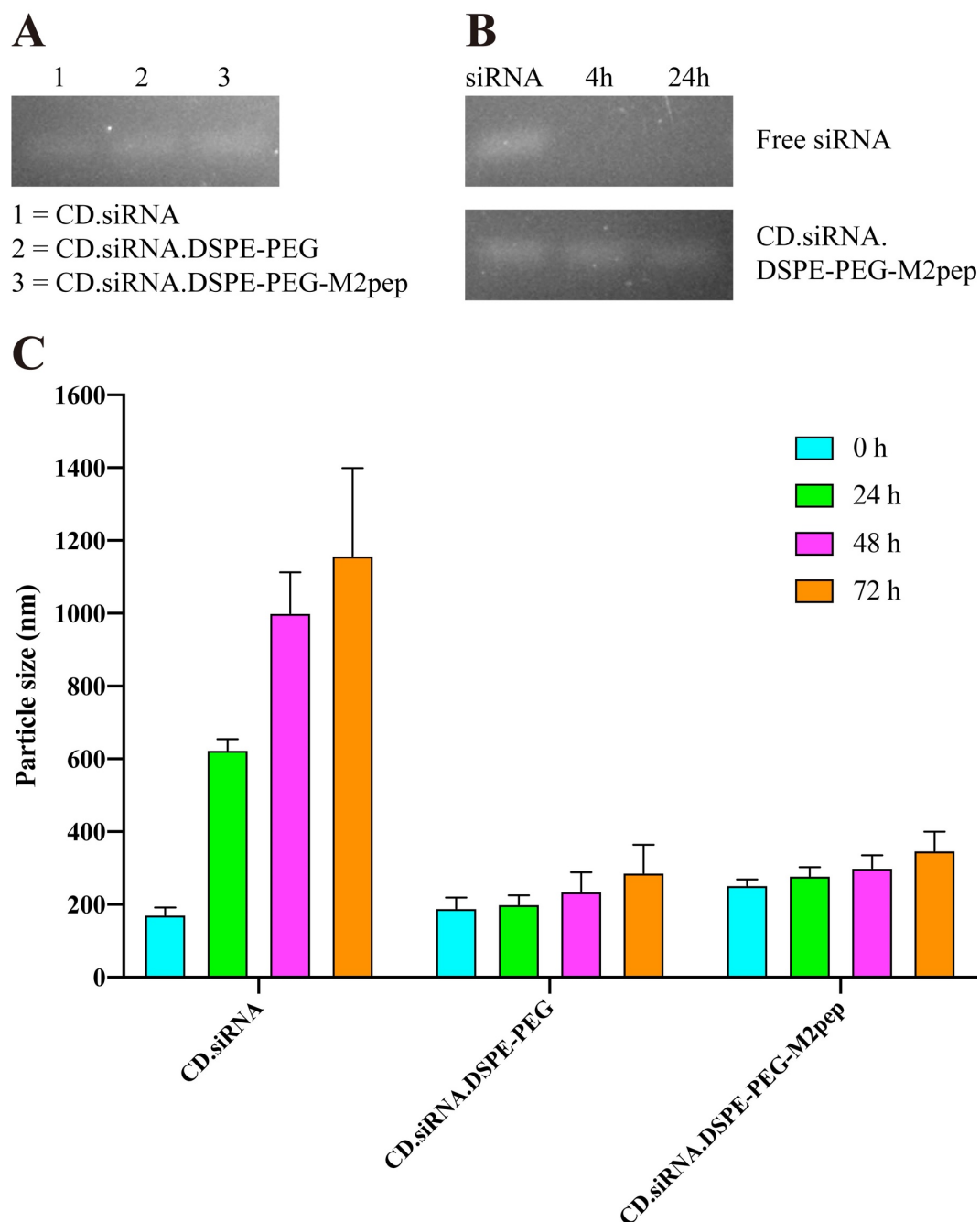


Fig. S3.4. (A) CD.siRNA, CD.siRNA.DSPE-PEG, and CD.siRNA.DSPE-PEG-M2 were treated with heparin (1000 I.U./mL) for 1 h at room temperature to release siRNA. siRNA was confirmed by using 1% agarose gel electrophoresis, the resulting gels were imaged under UV. (B) Stability of naked siRNA (0.5 μ g) and siRNA formulated with CD.DSPE-PEG-M2 after incubation for 4 h and 24 h in 50% FBS at 37 $^{\circ}$ C. After incubation, CD.siRNA.DSPE-PEG-M2 was treated with heparin (1000 I.U./mL) to

release siRNA. siRNA was confirmed by using 1% agarose gel electrophoresis, the resulting gels were imaged by UV. (C) Stability of CD.siRNA, CD.siRNA.DSPE-PEG, and CD.siRNA.DSPE-PEG-M2 in 90% Opti-MEM medium for 24 h, 48 h and 72 h (n = 6, mean $\pm$ SD). Particle sizes were measured by DLS.

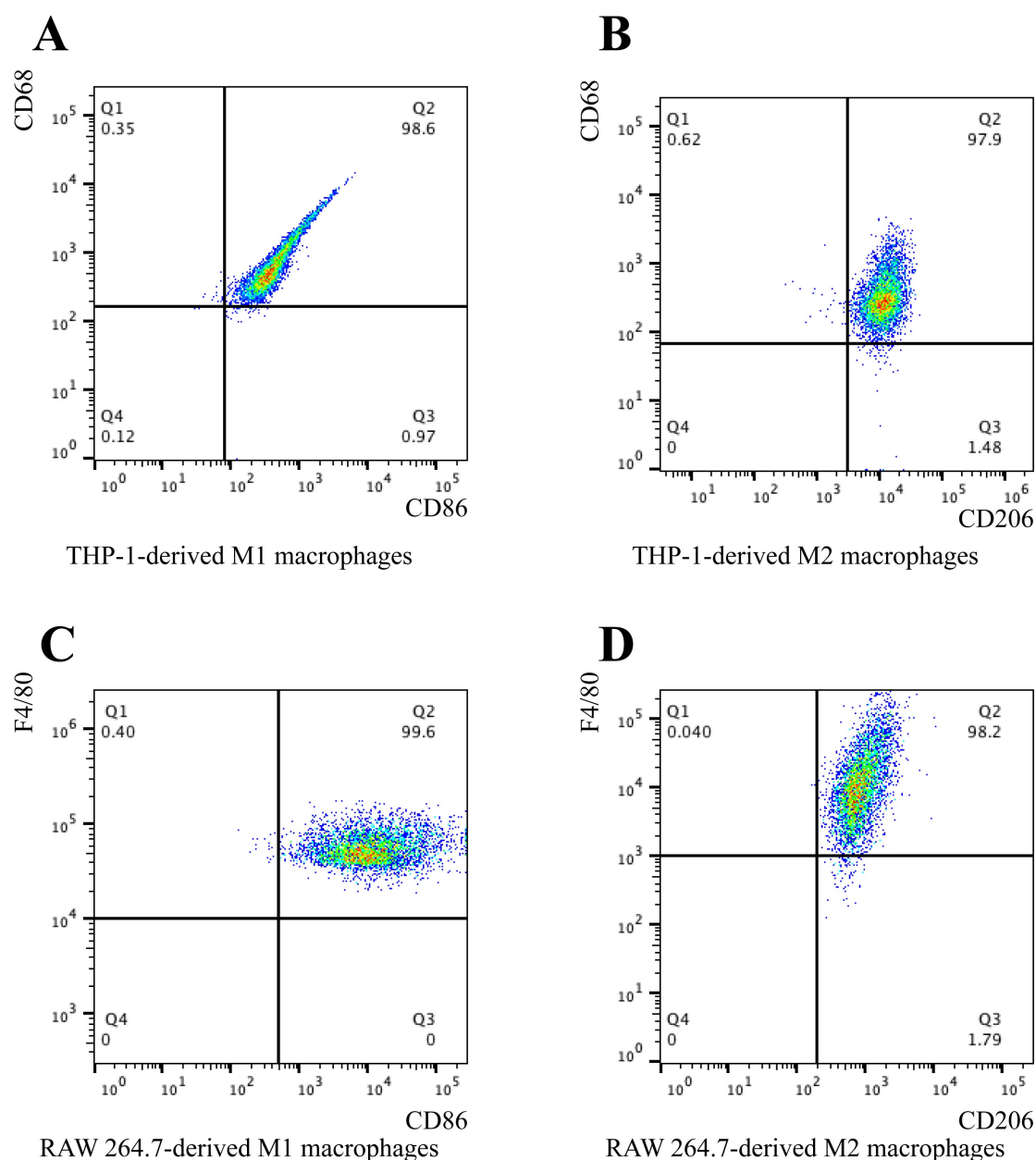


Fig. S3.5 The expression of human macrophages marker (CD68) and M1/M2 macrophage markers (CD86/CD206) on THP-1-derived M1 (A) or M2 macrophages (B); and the expression of mouse macrophages marker (F4/80) and M1/M2 macrophage

markers on RAW 264.7-derived M1 (C) or M2 macrophages (D), as measured by FACS.

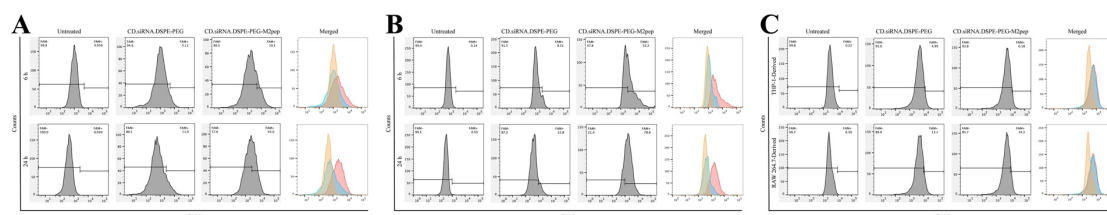


Fig. S3.6. Representative flow cytometry figures of cellular uptake of CD NPs on (A) THP-1- and (B) RAW 264.7-derived M2 macrophages after 6 h or 24 h transfection, and (C) competitive uptake study.

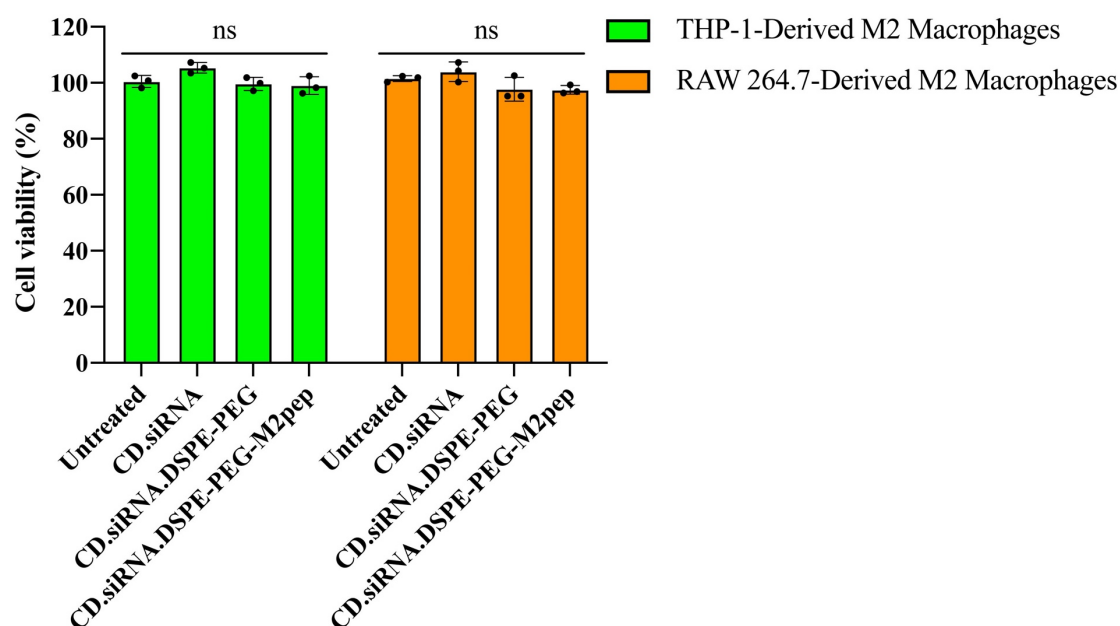


Fig. S3.7. THP-1- or RAW 264.7-Derived M2 macrophages were treated with different CD formulations for 24 h. Then CCK-8 was added with cells to measure cell viability (n = 3, mean $\pm$ SD, ns = not significant).

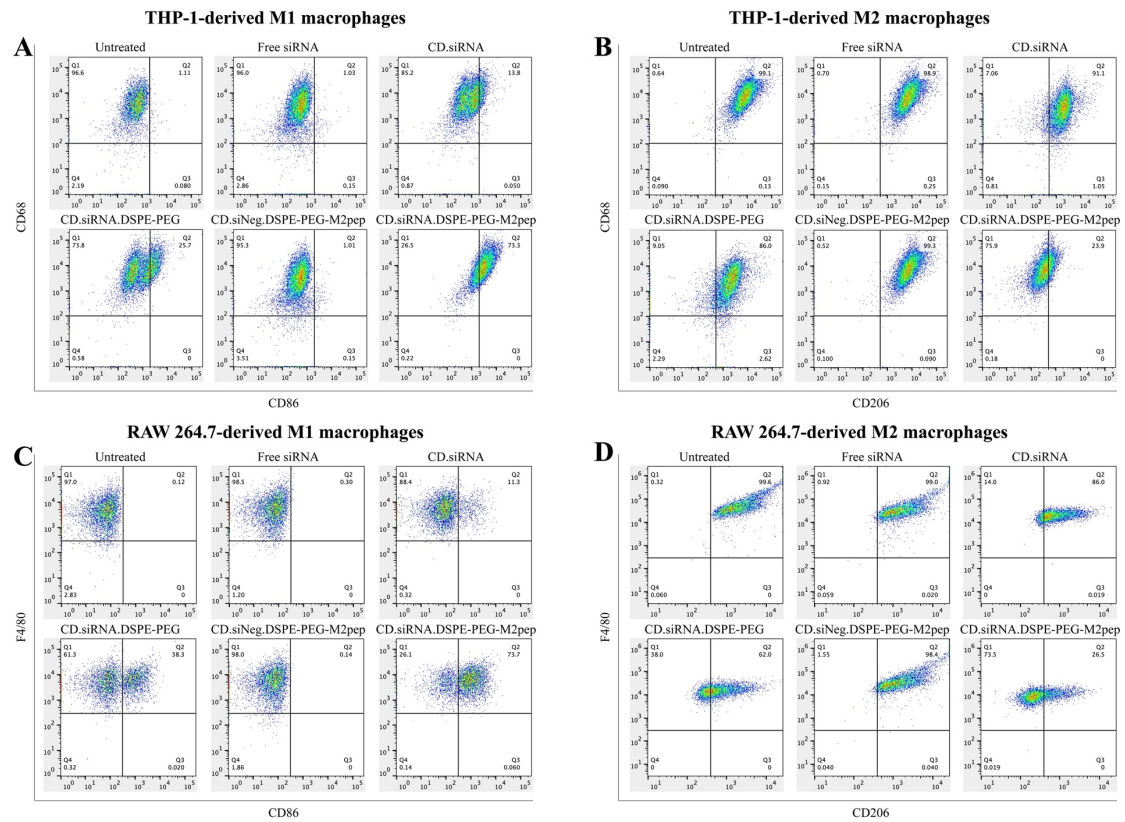


Fig. S3.8. Representative flow cytometry figures of cellular uptake of the expression of the human macrophage marker CD68 and M1/M2 marker (A) CD86/ (B) CD206 on THP-1-derived M2 macrophages, and the mouse marker F4/80 and M1/M2 marker (C) CD86/ (D) CD206 on RAW 264.7-derived M2 macrophages after transfection.

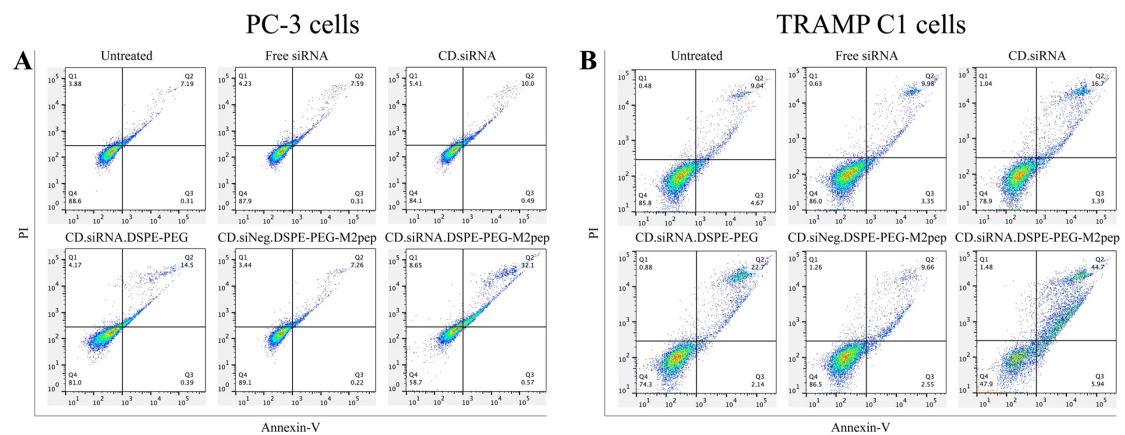


Fig. S3.9. Representative flow cytometry figures of (A) PC-3 and (B) TRAMP C1 cells apoptosis.

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Chapter 4 M2pep-Modified Cyclodextrin-siRNA Nanoparticles Modulate the Immunosuppressive Tumour Microenvironment for Prostate Cancer Therapy (*in vivo part*)

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4.1 Abstract

In the previous chapter 3, M2pep-targeted amphiphilic cyclodextrin (CD)-based NP incorporating siRNA NPs were characterised. Based on promising *in vitro* cell culture studies it was decided to test these NPs *in vivo* using a subcutaneous PCa mouse model. The resulting nanoparticles (NPs) increased M2 macrophage targeting *in vivo*, promoting the release of M1 factors and simultaneously downregulating the levels of M2 factors through TAM reprogramming. The subsequent remodelling of the TME resulted in a reduction in tumour growth in the mouse model mainly mediated through the recruitment of cytotoxic T cells. In summary, this M2pep-targeted CD-based delivery system demonstrated significant anti-tumour efficacy, thus presenting an alternative immunotherapeutic strategy for PCa treatment.

4.2 Keywords

Targeted nanoparticles, Functionalised Cyclodextrin; Tumour microenvironment, M2pep; M2 Macrophages; CSF-1R; Prostate Cancer; Immunotherapy

4.3 Introduction

As stated in section 3.3. in the current work, we are investigating the potential of M2pep-targeted amphiphilic cyclodextrin (CD)-based NPs to delivery siRNA for immunotherapy of PCa. Existing therapies for PCa are less than ideal and consequently there is an urgent need to develop new treatment approaches which are more effective and have lower risks of life altering side effects. In chapter 3, this formulation was

characterised and promising efficacy data was demonstrated *in vitro* using PCa cell models. Based on these initial results, the ability of the CD-siRNA NPs to reprogram the TAM was investigated in an *in vivo* PCa model using C57B/L6 mice.

4.4 Materials and Methods

4.4.1 Animals

Male C57 BL/6 mice (4-6 weeks) were purchased from Envigo (Bicester, UK). All animals were housed in Biological Service Unit (BSU), University College Cork, Ireland. The environment was specific pathogen-free and temperature-controlled. All animal experiments were approved by Animal Experimentation Ethics Committee of University College Cork (AEEC, UCC, No. UCC2021/022) and the Health Products Regulatory Authority (HPRA, No. AE19130/P159). All researchers were well-trained and authorized by HPRA for specified animal experiments.

4.4.2 In vivo tumour accumulation of NPs

A TRAMP C1 prostate carcinoma graft model was established by subcutaneous injection of 5.0×10^6 cells into 6-week-old male C57 BL/6 mice. Animal were divided into 3 groups ($n = 3$), including a free siRNA group, non-targeted, and targeted NPs groups. Body weight and tumour size were recorded every second day, tumour volume was calculated as follows:

$$V = \frac{L \times W^2}{2}$$

V = Volume of the tumour (mm³)

L = Longest dimension of the tumour (mm)

W = Shortest dimension of the tumour (mm)

When the tumour volume reached 100 mm³, the animals were treated with the different formulations containing Cy5-siNeg by intraperitoneal (i.p.) injection (Thaker et al., 2006, Merritt et al., 2008), the dose of siRNA was 1 mg/kg (Guo et al., 2012a). At 1, 2, 4, 6, and 24 h post injection, the animals were anesthetized by isoflurane and imaged using an IVIS® Spectrum system (IVIS Lumina, PerkinElmer, Waltham, MA, USA). Finally, the animals were euthanatized by cervical dislocation, organs including the heart, liver, spleen, and kidneys were collected and imaged using the IVIS® Spectrum system to quantify ex vivo the biodistribution of the NPs in the major organs.

4.4.3 In vivo anti-tumour effects

The TRAMP C1 prostate carcinoma model was built as described above. 30 mice were grouped into 6 groups randomly (n = 5). When the tumour volume reached 100 mm³, the mice were treated with the different formulations, the dose of siRNA was 1 mg/kg. Animals were treated by i.p. injection every second day on 5 occasions. At 10 days post final treatment, the animals were sacrificed by cervical dislocation. Major organs, including the heart, liver, spleen, lung, and kidneys, and tumours were collected and fixed in 10% formalin at 4 °C for 2 days. Then all tissues were transferred into 70% ethanol (v/v) and embedded in paraffin. All formalin-fixed paraffin-embedded (FFPE)

tissue blocks were cut into 5 μm thickness sections by a rotary microtome (HISTOCORE BIOCUT, Leica Biosystems, Wetzlar, Germany). Hematoxylin and eosin staining (H&E staining) was performed on organs and tumour sections, and terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL staining) was performed on tumour sections.

4.4.4 In vivo infiltration of T cells

The infiltration of T cells into tumour tissues was measured by immunofluorescence staining assay as follows. Tumour sections were incubated with FITC-anti-mouse CD3 antibody for 12 h at 4 °C, the nucleus was stained by DAPI and imaged by fluorescence microscopy. To identify CTLs and T helper cells, CD3 and CD8 or CD4 expression was measured by flow cytometry. Briefly, the tumour tissue was cut into small pieces and homogenized. Red blood cells were lysed and the cell suspension was stained with APC-CD3/PE-CD8 antibodies or APC-CD3/FITC-CD4 antibodies for 30 mins on ice, avoiding light, and measured by flow cytometry (n = 3). To determine the infiltration of regulatory T cells (Tregs), CD3+/CD4+ T helper cells were separated and then stain with a PE-CD25 antibody to measure the proportion of CD3+/CD4+/CD25+ cells by flow cytometry (n = 3). (Wu et al., 2018)

4.4.5 In vivo reprogramming of tumour-associated macrophages

To determine the *in vivo* reprogramming of TAMs, CSF-1R and M1/M2 factor expression in tumour tissue was measured by RT-qPCR, and M1/M2 cytokine expression was

measured by ELISA. The expression of M1 (CD86) and M2 markers (CD206) in tumour sections was determined by immunofluorescence staining. Sections were incubated with PE-anti-mouse CD86 and FITC-anti-mouse CD206 antibodies for 12 h at 4 °C and the nucleus stained by DAPI was imaged by fluorescence microscopy. To measure the proportion of M1 and M2 macrophages, cells were stained with APC-F4/80/PE-CD86 or APC-F4/80/FITC-CD206 antibodies and measured by flow cytometry (n = 3).

4.4.6 Statistical analysis

The statistical analysis was performed with Prism 8.0 (GraphPad, San Diego, CA, USA). Data were shown as mean $\pm$ standard deviation (mean $\pm$ SD) from at least 3 independent experiments. One-way analysis of variance (one-way ANOVA), followed by Dunnet or Tukey post hoc tests and Student's t-test was used to calculate the statistical significance between three or more groups and two groups. A p-value < 0.05 was considered a significant difference.

4.5 Results and Discussion

4.5.1 In vivo biodistribution of M2pep targeted NPs

A time course study indicated that tumour accumulation of NPs labelled with Cy5-siRNA was maximised at 2 h post injection (Fig. 4.1A). With the free siRNA group (Fig. 4.1A), only a weak signal was detected at 1 and 2 h with no signal monitored at other time points. In comparison, the targeted nanoparticles showed a significantly

higher tumour accumulation of Cy5-siRNA relative to other groups (Fig. 4.1B, $**p < 0.01$). The animals were euthanized after final imaging and major organs were collected, and ex vivo accumulation of Cy5-siRNA was monitored. Free siRNA was not detected in any organ. In contrast to the targeted NPs, the non-targeted NPs appeared to accumulate in the liver and kidneys (Fig. 4.1C & D), this is consistent with the lower Cy5-siRNA accumulation detected previously in tumours for the non-targeted NPs ($**p < 0.01$).

The results indicate that the formulation of siRNA within NPs increased the *in vivo* stability and avoided rapid degradation in the systemic circulation relative to naked siRNA, this is consistent with our *in vitro* stability results. Targeted NPs accumulated in the tumour area, in contrast non-targeted NPs displayed higher accumulation in the liver and kidneys, indicating the targeting efficiency of the M2pep ligand. When the same ligand was used to target pancreatic tumours (intravenous injection) a similar level (~2.4-fold versus non-targeted NPs at peak levels) of accumulation in the tumour tissue was detected albeit it that the time (4 h) to reach peak levels was later.(Li et al., 2020)

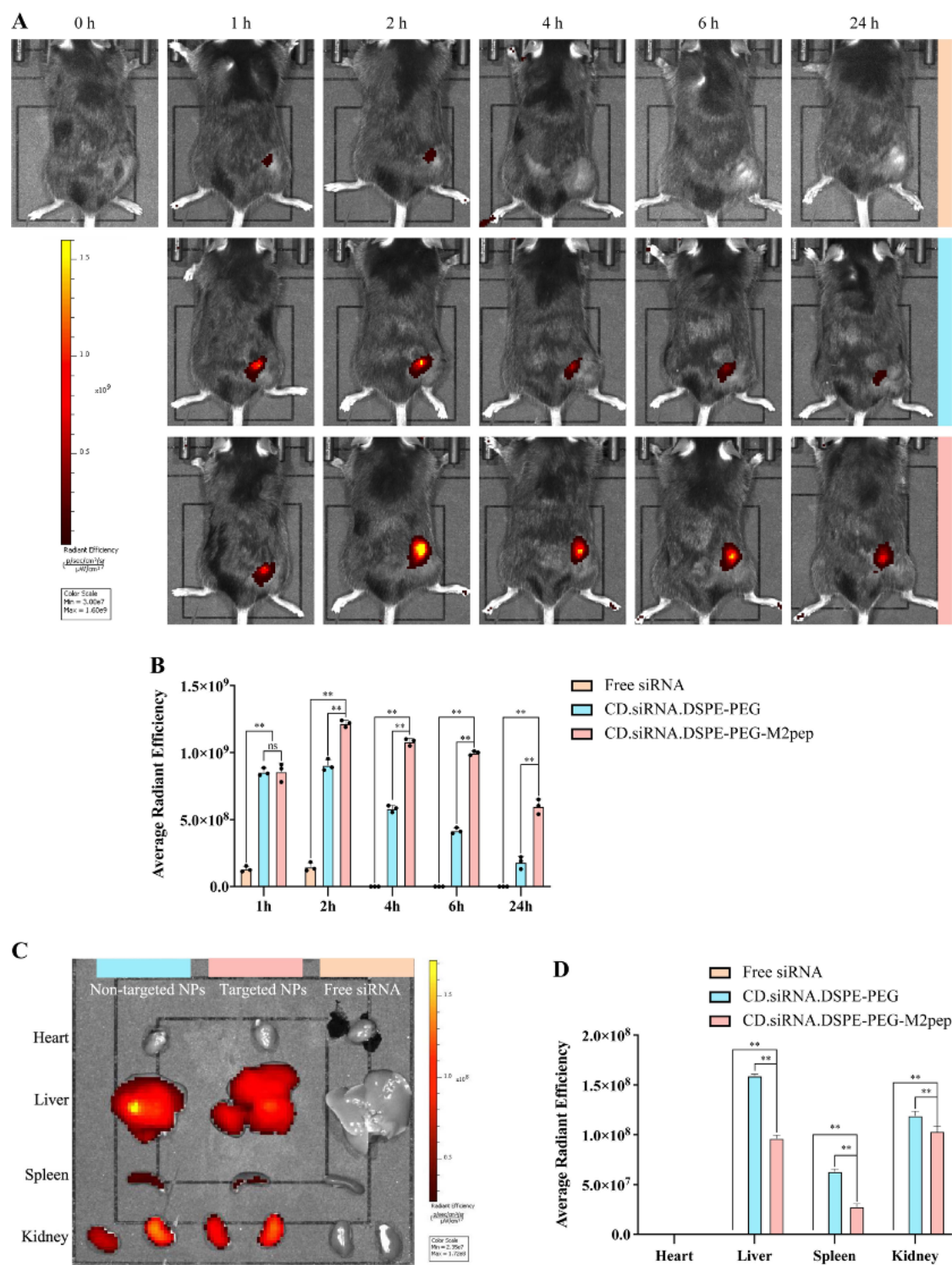


Fig. 4.1. (A) *In vivo* biodistribution of (i) free siRNA (top line, orange); (ii) CD.siRNA.DSPE-PEG (middle line, blue); and (iii) CD.siRNA.DSPE-PEG-M2pep (bottom line, pink) labelled with Cy5-siRNA at 1h, 2h, 4h, 6h, and 24h after injection. (B) Semi-quantitative analysis of tumour accumulation ($n = 3$, ns = not significant,

mean $\pm$ SD, $**p < 0.01$). (C) *Ex vivo* biodistribution of Cy5-siRNA 24 h post-injection in organs of different formulations. (D) Semi-quantitative analysis of tissue biodistribution (n = 3, mean $\pm$ SD, $**p < 0.01$).

4.5.2 *In vivo anti-tumour effects*

The *in vivo* anti-tumour effects were analyzed in a TRAMP C1 prostate carcinoma mouse model following i.p. administration. Following treatment with the targeted NPs containing siRNA against CSF-1R, both tumour volumes (Fig. 4.2A & B) and weights (Fig. 4.2C) significantly decreased (75.88% in tumour size decrease, and 75.90% in tumour weight decrease, compared with untreated group $**p < 0.01$), compared with other groups. During the study, the body weight of tumour-bearing animals increased gradually in all cases suggesting no toxicity due to the formulations administered (Fig. S4.1).

On day 39, the animals were euthanatized, H&E staining was performed on the tumour (Fig. 4.2D) and organ sections (Fig. S4.2), and TUNEL staining was performed on tumour sections (Fig. 4.2E). The tumour section staining results indicated that the targeted NPs promoted higher levels of apoptosis relative to controls (Fig. 4.2F, $**p < 0.01$), which is consistent with the tumour volumes and weights results. Examination of the slides from the major organs did not show any evidence of toxicity.

In summary, the CD-based M2pep-targeted CSF-1R siRNA NPs achieved a significant reduction in, tumour volumes (75.88%) tumour weights (75.90%) compared with untreated groups. These results compare well with previously published data, where 1,2-dimyristoyl-sn-glycero3-phosphocholine (DMPC)- and cholesterol oleate (C.O)-based M2pep/ α -peptide-dual targeted NPs containing CSF-1R siRNA reduced the tumour size by 87% in melanoma-bearing animal models.(Qian et al., 2017) In addition, a tumour size reduction of (73%) was observed in an M2pep-targeted polyethylenimine-stearic acid-based NPs with CSF-1R siRNA and PI3K- γ inhibitor in a pancreatic cancer model, while the NPs containing only CSF-1R siRNA achieved around a 50% reduction in tumour size.(Li et al., 2020)

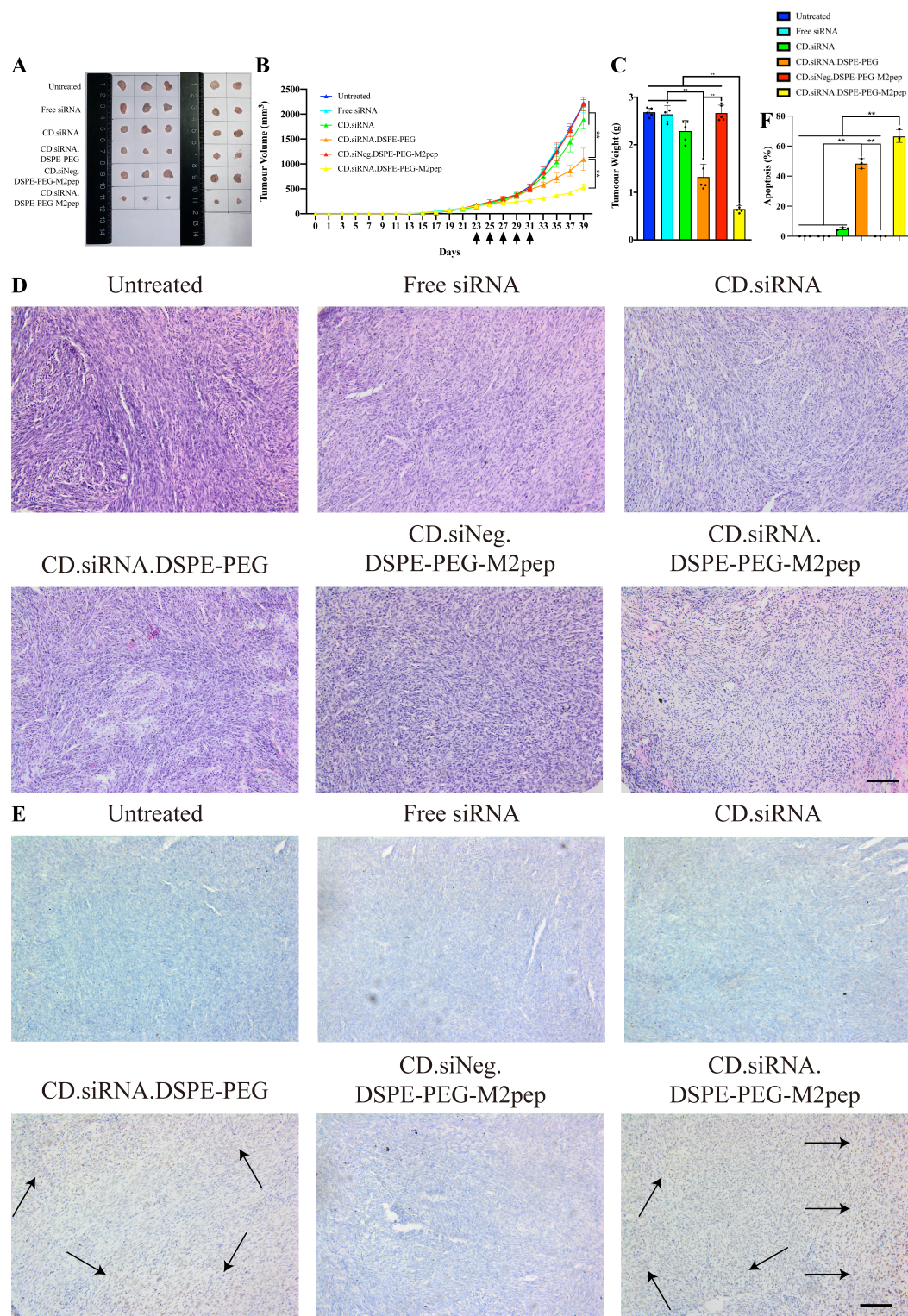


Fig. 4.2. (A) Images of tumour tissues from the prostate cancer-bearing models on day 39. (B) Tumour volumes over the course of the study following i.p administration on D23, D25, D27, D29, D31 (black arrows) of NPs with siRNA against CSF-1R (D39: $n = 5$, mean $\pm$ SD, $**p < 0.01$). (C) Tumour weight from the prostate cancer-bearing

models on day 39 ($n = 5$, mean $\pm$ SD, $**p < 0.01$). (D) H&E staining of tumour sections for controls and NP treatments. (E) TUNEL staining of tumour sections for controls and NP treatments (black arrow: apoptosis areas), haematoxylin was used for counterstaining the nucleus. Scale bar = 100 μ m. (F) the level (%) of apoptosis in the tumour sections ($n = 3$, mean $\pm$ SD, $**p < 0.01$).

4.5.3 In vivo remodeling of tumour microenvironment

To investigate the mechanism of the anti-tumour effects and the remodeling of the TME, T cell infiltration in the tumours was monitored. Following immunofluorescence staining a higher T cell level was observed after treatment with the targeted NPs (Fig. 4.3A). Different types of T cells were separated by flow cytometry, CTLs were CD3⁺/CD8⁺ (Fig. 4.3B), and T helper cells (Th cells) were CD3⁺/CD4⁺ (Fig. 4.3C). The results indicated that the targeted NPs recruited significantly more T cells in the tumour area relative to other treatment groups (Fig. 4.3E - F, $**p < 0.01$). The upregulation of T cell infiltration is a key sign of TME remodelling, the targeted NPs increased CD8⁺ CTLs 3.8-fold versus the untreated group. A similar trend has been reported where M2pep-targeted polyethylenimine-stearic acid-based NPs simultaneously containing CSF-1R siRNA and NVP-BEZ 235 (a PI3K- γ inhibitor) increased the levels of CD8⁺ CTLs 1.5-fold compared with the untreated group in a pancreatic cancer animal model, while the M2pep-targeted NP containing CSF-1R siRNA produced a 1.1-fold increasing in the CD8⁺ CTL level.(Li et al., 2020) In a further study, NPs formulated with DMPC and C.O containing CSF-1R siRNA with an

M2pep and α -peptide dual ligand achieve a 2.7-fold higher CD8⁺ CTL level in a melanoma-bearing mouse model.(Qian et al., 2017)

The proportion of regulatory T cells (Tregs) within the Th cells was monitored by flow cytometry using CD25 as a Treg marker for detection in the CD3⁺/CD4⁺ populations. The results show that the proportion of Tregs following treatment with the targeted NPs was significantly lower than the other groups (Fig. 4.3D & G) (** $p < 0.01$) suggesting that the targeted formulation downregulated the immunosuppressive T cells in the tumour while the higher infiltration of CD8⁺ CTLs marked the successfully remodelled TME. This trend is consistent with the anti-tumour effects described above.

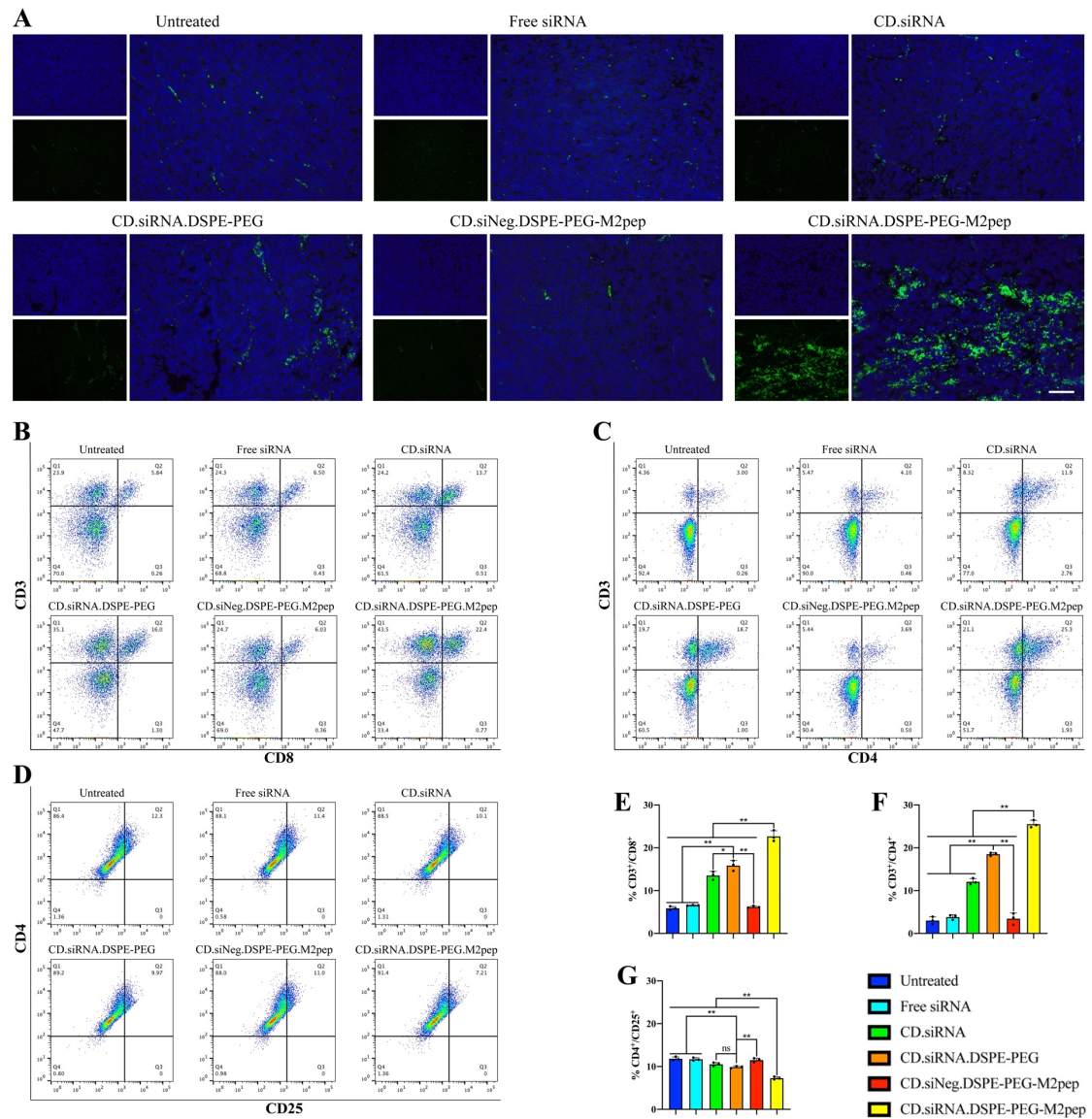


Fig. 4.3. (A) Immunofluorescence of T cells infiltration in tumour sections. Green: FITC-CD3. Blue: DAPI. Scale bar = 100 μ m. (B) Cytotoxic T cells (CD3+/CD8+) infiltration in tumour tissues. (C) T helper cells (CD3+/CD4+) infiltration in tumour tissues. (D) Regulatory T cells (CD3+/CD4+/CD25+) infiltration in tumour tissues. (E) Percentage of cytotoxic T cells (CD3+/CD8+) infiltration (n = 3, mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$). (F) Percentage of T helper cells (CD3+/CD4+) infiltration (n = 3, mean $\pm$ SD, ** $p < 0.01$). (G) percentage of Regulatory T cells (CD3+/CD4+/CD25+) infiltration in tumour tissues (n = 3, ns = not significant, mean $\pm$ SD, ** $p < 0.01$).

4.5.4 In vivo reprogramming of TAMs

As the upregulation of immunostimulatory cytokines like IL-12 can promote CTL activation, RT-qPCR and ELISA were performed to measure both M1 and M2 factor expression in tumour samples. As the data above indicate TME remodeling and T cell infiltration play significant roles promoting the anti-tumour effects, as both are caused by the reprogramming of TAMs the later was subsequently measured. Treatment with the targeted NPs silenced the expression of CSF-1R at the mRNA level (Fig. 4.4A) resulting in significant downregulation of M2 factors (Fig 4.4E - H) and upregulation of M1 factors (Fig. 4.4B - D) relative to other treatment groups (* $p < 0.05$, ** $p < 0.01$). These results were confirmed at the protein expression level as shown by ELISA (Fig. 4.4I - J).

In addition, the expression of M1/M2 macrophage markers was tested via immunofluorescence and flow cytometry to analyse TAMs phenotypes. The M1 marker, CD86, and the M2 marker, CD206 in tumour sections, were monitored by immunofluorescence staining (Fig. 4.5A). Results suggested that the M1 marker was higher in the targeted nanoparticles group with a lower M2 marker level. This trend was confirmed by flow cytometry measuring the proportion of M1 macrophages (F4/80+/CD86+) and M2 macrophages (F4/80+/CD206+) (Fig. 4.5B & C). More M2 macrophages were reprogrammed to the M1 phenotype following treatment with the targeted nanoparticles compared to the other treatment groups (** $p < 0.01$) (Fig. 4.5D

& E).

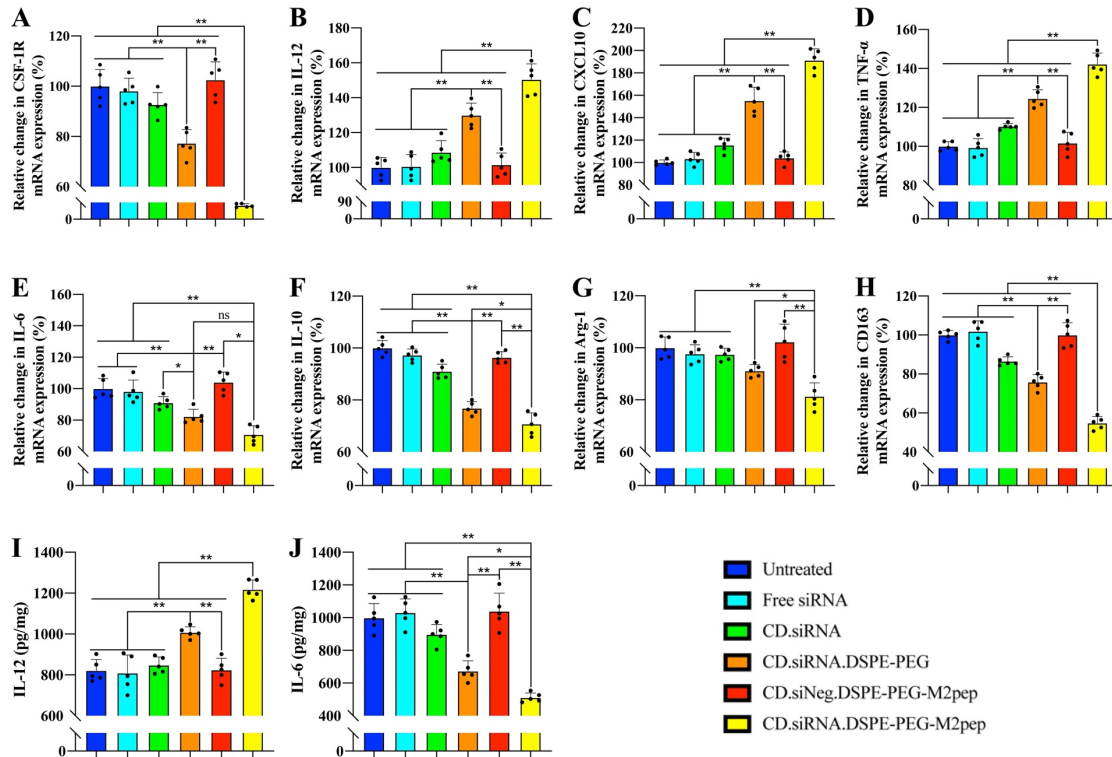


Fig. 4.4. (A) *In vivo* downregulation of CSF-1R by formulations containing CSF-1R siRNA. (B - D) Upregulation of M1 factors by CD formulations at the gene level, including IL-12, CXCL10, and TNF- α . (E - H) Downregulation of M2 factors by CD formulations at the gene level, including IL-6, IL-10, Arg-1, and CD163. (I - J) Protein levels of IL-12 and IL-6 (n = 5, ns = not significant, * p < 0.05, ** p < 0.01).

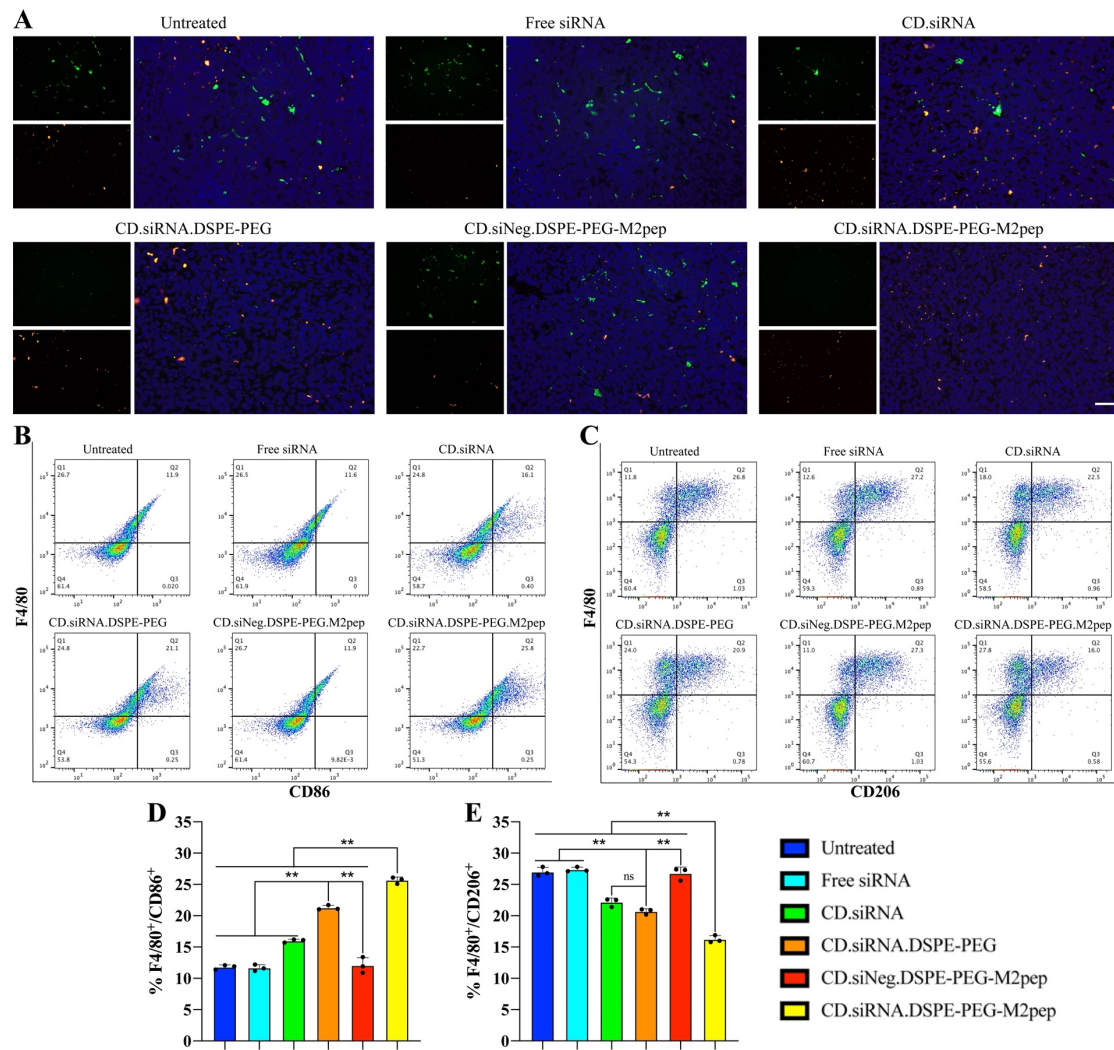


Fig. 4.5. (A) Immunofluorescence of reprogramming of TAMs. Blue: DAPI, green: FITC-CD206, and red: PE-CD86. Scale bar = 100 nm. (B) M1 macrophages (F4/80⁺/CD86⁺) level in tumour tissues. (C) M2 macrophages (F4/80⁺/CD206⁺) level in tumour tissues. (D) Percentage of M1 macrophages (F4/80⁺/CD86⁺) and (E) Percentage of M2 macrophages (F4/80⁺/CD206⁺). (n = 3, ns = not significant, **p* < 0.05, ***p* < 0.01).

In summary, as PCa is an immunosuppressive cancer an immunotherapeutic approach provides an alternative strategy to design more clinically effective treatments. The CSF-

1/CSF-1R pathway has been reported as a potential target for cancer immunotherapy. Downregulation of CSF-1R by inhibitors, antibodies or siRNA has been shown to limit tumour growth in a variety of tumour models.(Achkova and Maher, 2016, Kumari et al., 2018) The repolarization of TAMs mediated this tumour limitation by silencing the CSF-1/CSF-1R pathway. The repolarised M1 macrophages release M1 factors including cytokines (IL-12, TNF- α , etc.) and chemokines (CXCL10, etc.) to recruit CD4⁺/CD8⁺ cytotoxic T cells to the tumour area to directly kill tumour cells. At the same time, the downregulation of the M2 macrophage level leads to a reduction in the recruitment of immunosuppressive cells, such as MDSCs and Tregs. The higher level of immunostimulatory cell recruitment and the lower level of immunosuppressive cell recruitment causes the remodelling of TME which subsequently achieves PCa immunotherapy.(Lesokhin et al., 2012, Franklin et al., 2014, Mantovani et al., 2017, Pathria et al., 2019)

To enable clinical translation of siRNA an effective and safe delivery system is essential. In the current study, a CD-based NP was designed for CSF-1R siRNA delivery with an M2pep-targeting ligand chosen to specifically target M2 macrophages. Both *in vitro* and *in vivo* data show the targeted NPs achieved CSF-1 gene knockdown resulting in cell apoptosis in the PCa cell models and inhibition of tumour growth in the animal model. Mechanistic studies indicated that the anti-tumour efficacy was due to high T cell infiltration, including TC cells, and Th cells, promotion of higher levels of M1 macrophages and a simultaneous decrease in M2 macrophage levels.

The immunotherapy approach, exploited in this work, is based on remodelling the TME landscape by reprogramming M2 macrophages to the M1 ones. Functionalised CDs have previously been used successfully for siRNA delivery in cancer gene therapy.(Guo et al., 2012b, Evans et al., 2017) The flexibility of the CD ring structure facilitates the covalent attachment of various functional groups, such as cationic groups to bind the negatively charged siRNA. In addition, the attachment of the lipid component results in a self-assembling amphiphilic CD which can simultaneously increase membrane permeability and facilitate the co-formulation, via post insertion, with other lipid entities in this case the DSPE-PEG-M2pep targeting ligand. The resulting CD-based targeted nanoparticles have shown efficacy in the *in vivo* PCa mouse model by successfully remodelling the TME.

In addition to the formulation approach used in this work, CDs as biomaterials have the added bonus of providing an opportunity to incorporate small lipophilic anti-cancer drugs within the hydrophobic cavity, by inclusion complex formation, thus facilitating a combination therapy approach using the same delivery system with the potential for stronger anti-tumour efficacy.(Zou et al., 2021, Sun et al., 2022)

4.6 Conclusion

In the current study, a smart drug delivery system was constructed to deliver CSF-1R siRNA. The specific targeting efficacy for M2 macrophages was verified *in vivo*. In

addition, the NPs reprogrammed M2 macrophages through silencing CSF-1R mRNA expression, in turn the reprogrammed TAMs remodelled the TME, reactivating the infiltration of CD4⁺/CD8⁺ T cells while decreasing the Tregs level, culminating in PCa cells apoptosis in a PCa bearing mouse model. While the application of gene therapy-based immunotherapy in PCa has previously been explored *in vitro* (Sun et al., 2023), to our knowledge this is the first report describing *in vivo* efficacy thus illustrating the potential to translate this strategy for prostate cancer therapy.

4.7 CRediT authorship contribution statement

Yao Sun: Methodology, Formal analysis, Writing – original draft. Michael F. Cronin: Methodology, Formal analysis, Writing – review & editing. Monique C.P. Mendonça: Resources, Writing – review & editing. Jianfeng Guo: Conceptualization, Supervision, Writing – review & editing. Caitriona M. O’Driscoll: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

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4.9 Supporting Information

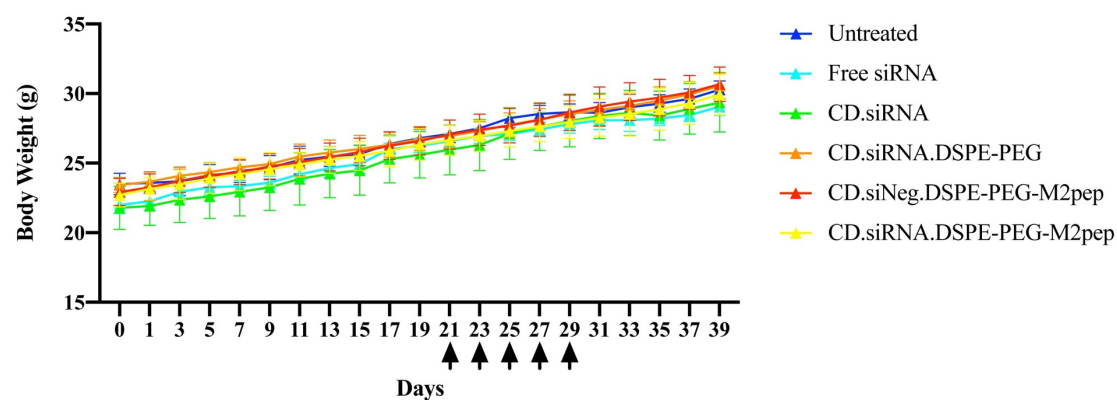


Fig. S4.1. Body weight changes of prostate cancer-bearing models during anti-tumour effects study (n = 5, mean $\pm$ SD).

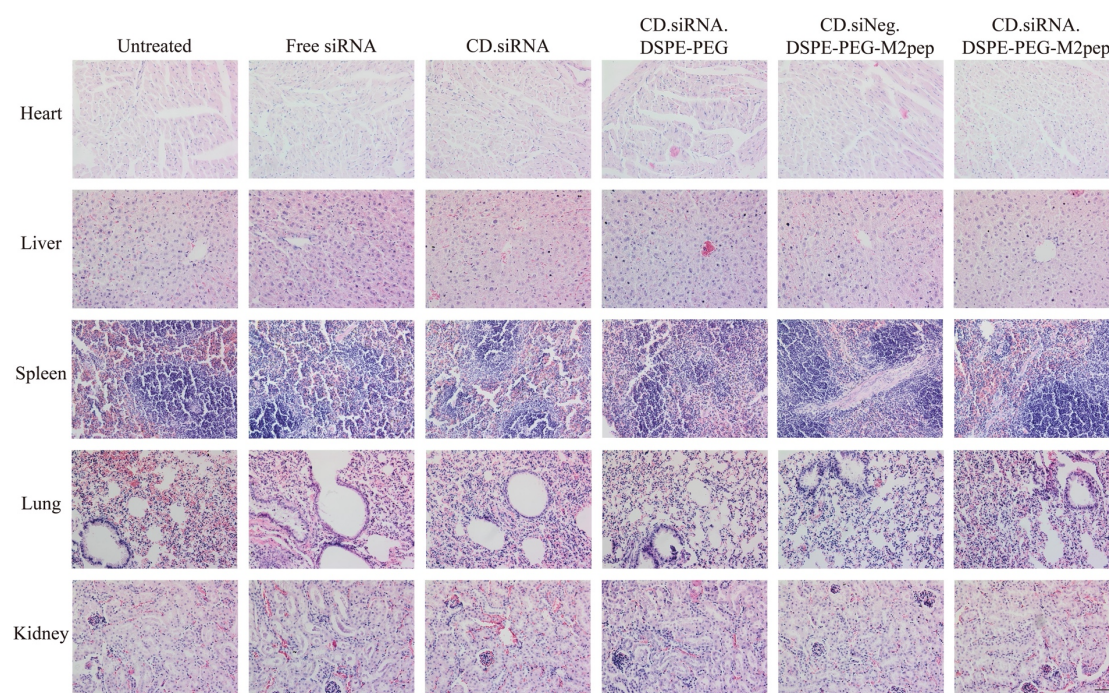


Fig. S4.2. H&E staining of major organs sections among 6 groups. Scale bar = 50 μ m.

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Chapter 5 General Discussion

5.1 Overview & Summary

Different from early-stage prostate cancer, advanced prostate cancer as an immunosuppressive cancer is difficult to manage by conventional treatment, therefore new therapeutic strategies are urgently needed. In recent years, immunotherapy achieved by activating the autoimmune system has shown great promise in the treatment of a range of cancers including pancreatic, colon, breast cancer, melanoma and prostate cancer (Sun et al., 2022, Zou et al., 2021, Li et al., 2020, Qian et al., 2017, Sun et al., 2023). This treatment approach focuses on remodelling the immunosuppressive tumour microenvironment, converting it into an immune-activating type, thereby promoting the accumulation of cytotoxic T cells at the tumour site to achieve inhibition of solid tumours. Immunotherapy is a targeted approach for treating malignant tumours, which has shown tremendous potential. The principle of immunotherapy is to use patients' own immune system to combat tumour cells. Immunotherapy includes monoclonal antibodies, immune checkpoint inhibitors, therapeutic antibodies, cell therapy, and more, demonstrating outstanding efficacy and innovation (Cao and Kagan, 2023). After the successful use and results of immune checkpoint inhibitors (ICIs) targeting programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1), and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), immunotherapy has been widely noticed by the patients and doctors (Topalian et al., 2023).

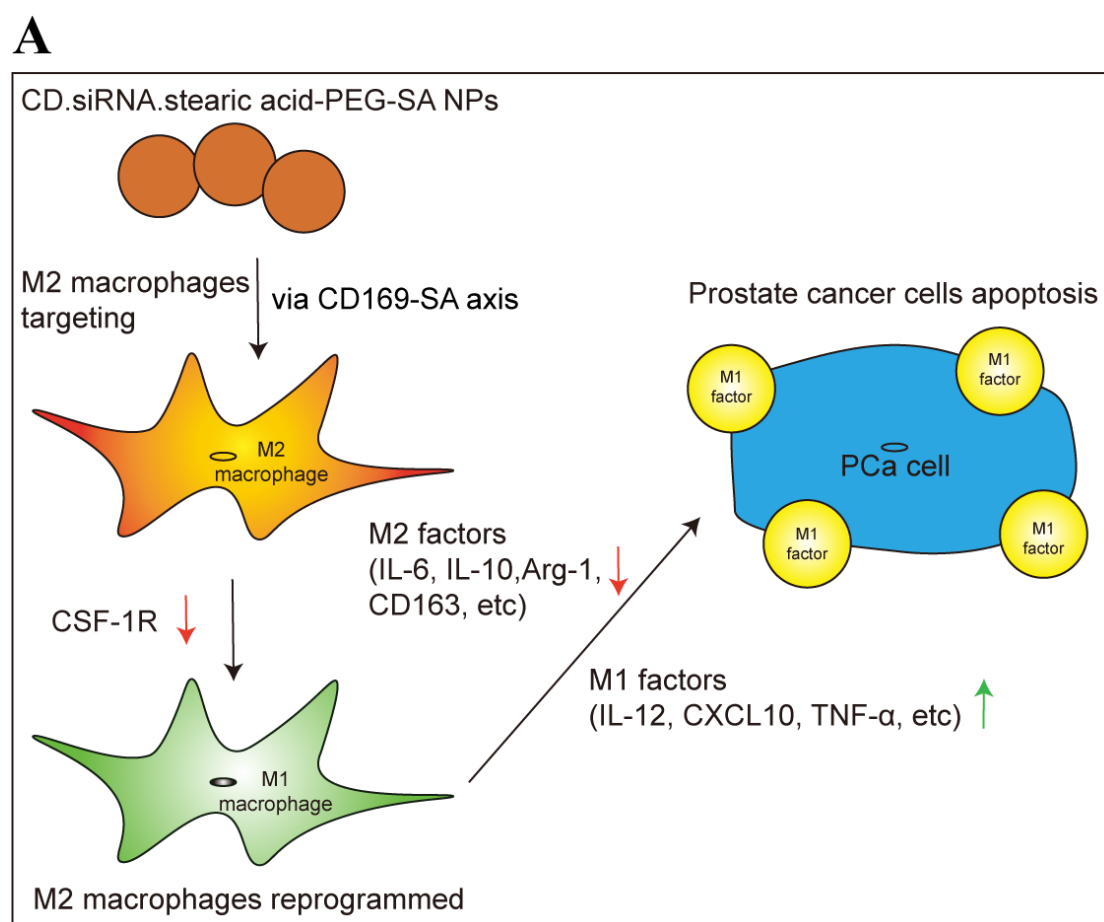
However, during the treatment process, a significant portion of patients with solid tumours still do not respond to or develop resistance to ICI therapy. Therefore, researchers have been tirelessly exploring new immune targets. Including macrophage-targeted drugs. Some potential targets for macrophage targeting have shown rapid clinical progress, including the CSF-1/CSF-1R pathway, SIRP α -CD47 pathway, and CD40 (Majety et al., 2018).

In this thesis, cyclodextrin-based drug delivery systems were designed for siRNA delivery to M2 macrophages to achieve prostate cancer immunotherapy. Compared to the conventional treatment strategies which are effective for early-stage prostate cancer (including Stages I to III), immunotherapy has advantages such as bare resistance, high therapeutic effectiveness, and low side effects, which make it more suitable for the treatment of advanced prostate cancer (Stage IV).

Different nanoparticles based on cationic amphiphilic beta-cyclodextrin were designed for delivering anti-colony stimulating factor-1 receptor siRNA (CSF-1R siRNA) to M2 macrophages to reprogram these immunosuppressive tumour-associated macrophages to an immunostimulatory phenotype. These nanoparticles specifically target M2 macrophages via the sialic acid-CD169 axis and M2pep-M2 macrophage axis.

Figure 5.1 shows the anti-PCa mechanisms of 2 cyclodextrin-based formulations. The

stearic acid-PEG-SA-based NPs containing CSF-1R siRNA reprogrammed M2 macrophages via downregulation of CSF-1R expression, subsequently, the M1 factors (IL-12, CXCL10, TNF- α , etc) released by M1 macrophages initiated the anti-PCa effect (Fig. 5.1A, Chapter 2), and M2pep-based NPs containing CSF-1R siRNA in a PCa-bearing mouse model, NPs reprogrammed M2 macrophages to the M1 phenotype, then release M1 factors to recruit cytotoxic T cells to the tumour area to achieve the anti-PCa effect *in vivo* (Fig. 5.1B, Chapter 4).



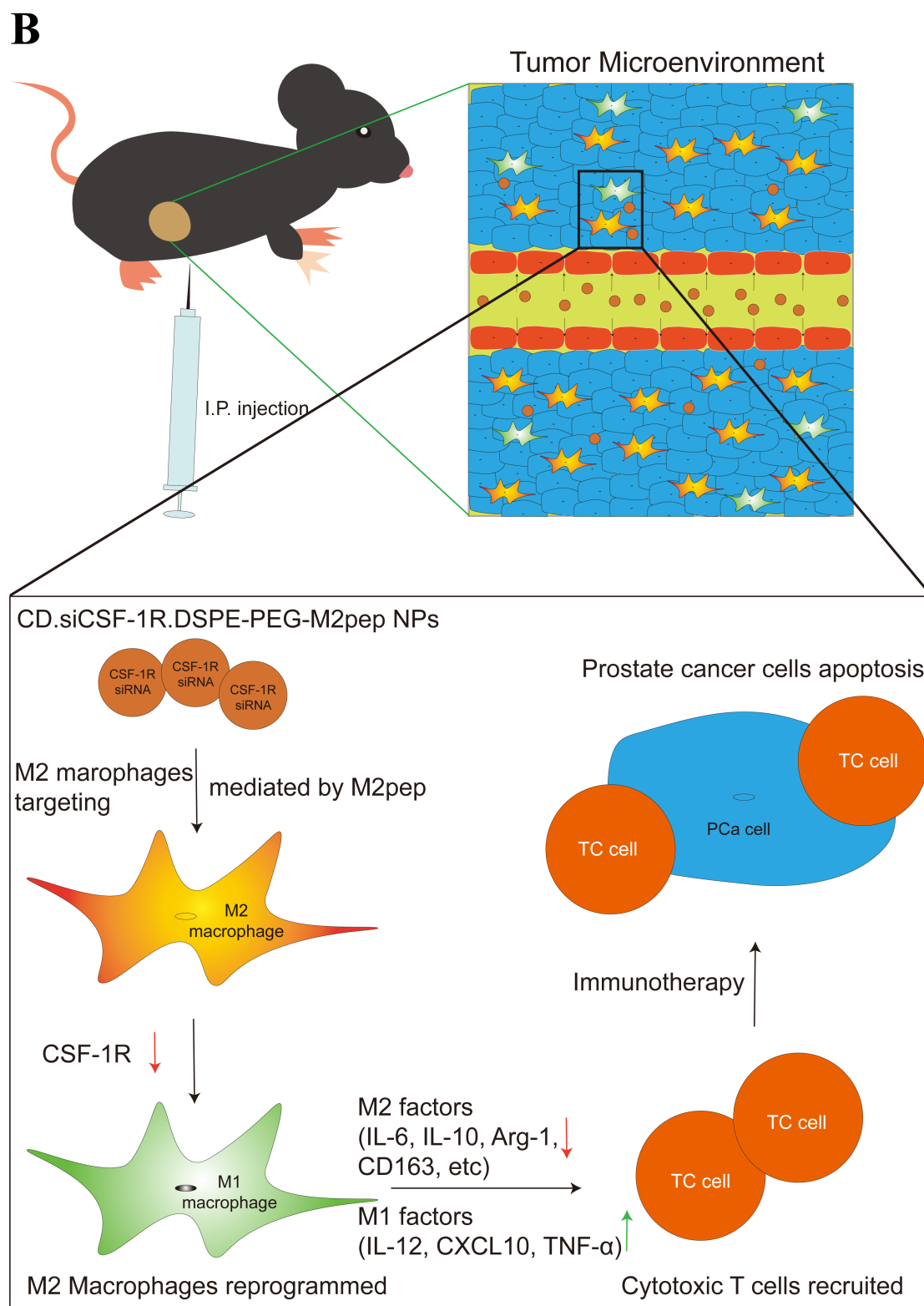


Fig. 5.1 Graphical abstracts illustrating the anti-tumour mechanism of CD-based siRNA NPs.

The *in vitro* studies showed that all nanoparticles investigated silenced CSF-1R mRNA expression, reprogramed TAMs from M2 to M1 phenotype, upregulated the secretion of M1 factors, such as IL-12, TNF-alpha, and in contrast downregulated the secretion of M2 factors (IL-6, Arg-1). In a tumour microenvironment *in vitro* model simulated by co-culturing macrophages and prostate cancer cells, CD-based nanoparticles promoted PCa cell apoptosis. While both sialic acid- and M2pep-targeted NPs successfully targeted M2 macrophages, compared with the sialic acid-targeting ligand, the M2pep-based NPs presented a higher cellular uptake in the M2 macrophages. The ligand construct for the M2Pep was DSPE-PEG-M2pep.

In order to increase the efficacy of PCa immunotherapy, M2pep, the second targeting ligand was investigated. Of the 2 formulations, the M2pep ligand produced the highest uptake levels and the highest CSF-1R gene silencing effects, which were 42.2% and 57.7% in THP-1 and RAW 264.7-derived M2 macrophages, ($p < 0.01$). In addition, a higher degree of M2 macrophages reprogramming was detected (73.0% and 71.9% of THP-1 and RAW 264.7-derived M2 macrophages), this translated into 31.4% apoptosis in PC-3 cells and 43.6% in TRAMP C1 cells (Chapter 3).

It is noteworthy that results from the mouse cells, RAW 264.7-derived M2 macrophages, were in general better than results from human cells, THP-1-derived M2 macrophages. This may be due to the fact that RAW 264.7 cells are original macrophages, while THP-1 cells are monocytes and require two steps to differentiate them into M2 macrophages.

This may affect cell proliferation making RAW 264.7 cells more sensitive to NPs.

However, this requires further investigation.

Based on the promising *in vitro* data a decision was made to test the M2pep targeted NPs *in vivo*. A PCa-bearing mouse model was established by subcutaneous injection of the mouse PCa cell line TRAMP C1 in male C57 BL/6 mice and used to explore the targeting and anti-tumour effects of the M2pep ligand. Results show that the targeted NPs accumulated significantly in the tumour area (up to 2.2-fold higher than the non-targeted formulation), reprogramed and remodelled the TAMs and the TME, and recruited cytotoxic T cells into the tumour area, all resulting in a reduction in PCa tumour growth (up to a 2-fold reduction in the tumour size/weight relative to the non-targeted NPs, and 5-fold reduction relative to the untreated group, Chapter 4).

These results demonstrate the potential of immunotherapy in prostate cancer. Compared to targeting PCa cells themselves, immunotherapy aimed at the immune cells can initiate an immune response resulting in tumour inhibition. In addition, the advantages of cyclodextrins including low toxicity, high transfection efficiency, and the ability to add functional moieties via the hydroxyl groups are major advantages making CDs an attractive option as a non-viral nucleic acid delivery vector. (O'Mahony et al., 2013, Haley et al., 2020).

5.2 Advantages and Limitations of Immunotherapy

In summary, this thesis introduces a new therapeutic method based on M2 macrophages reprogrammed targeting advanced prostate cancer. This new immunotherapy approach differs from traditional treatments by reprogramming M2 macrophages, remodelling the tumour microenvironment, and recruiting cytotoxic macrophages to target and kill prostate cancer cells. Compared to conventional treatments such as radiation therapy and surgery, immunotherapy focuses on the immune system with low side effects and a better prognosis. In addition, given that this new treatment does not involve the direct killing of tumour cells, but modulates toxic cytotoxic T cells through the tumour-associated macrophages, there is no issue of tumour resistance typically seen in the later stages of radiotherapy or chemotherapy.

However, as a novel developed therapy, immunotherapy still faces some limitations. First is the unpredictable efficacy, the majority of immunotherapy treatments have been only effective in a small subset of malignant growths (Yang, 2015). Additionally, many changes identified in human growth do not occur consistently across many patients. Immunotherapies need indeed be modified and patient-explicit to be more efficacious (Zugazagoitia et al., 2016). Furthermore, immunotherapy is typically administered to individuals whose immune systems have been impaired by a recent illness and previous treatments because they have not yet been extensively demonstrated as first-line therapy. Higher survival rates may be achieved in more patients on the off chance that personalised malignant growth immunotherapies were previously managed to re-establish a potent anti-tumour reaction. At the same time, the immune system can still

recover. The ability of disease immunotherapies to re-establish antitumour-resistant capability under these circumstances is testing (Schumacher and Schreiber, 2015).

The second limitation is immune toxicity. Immunotherapy is constrained by its immune-related adverse events (irAEs), impervious actuation, and incendiary reactions to the healthy tissues (Wu and Cai, 2021). It is reported that the growth of a CTLA-4 immune response to PD-1 blockade in the context of metastatic melanoma is associated with a slow increase in endurance but at the cost of more than twice the rate of significant irAEs (Chiriva-Internati and Bot, 2015). Therefore, modulating and predicting immune toxicity is essential to the development of immunotherapy.

The next limitation is cost-effectiveness, in previous studies, it has been reported that the cost-effectiveness of immunotherapy relies on the therapeutic signs, the usage of biomarkers, and the ligands used for the delivery vectors (Green, 2021, Rescigno et al., 2007).

The final and biggest limitation of immunotherapy is the existence of the immunosuppressive tumour microenvironment. Persistent antigen exposure in the tumour microenvironment causes CD8⁺ T cells to become depleted or damaged. These damaged or depleted cytotoxic T cells exhibit distorted cytokine production and proliferative limitations. At the same time, these damaged T cells upregulate inhibitory receptors (IRs), like PD-1, CTLA-4, T cell immunoglobulin and mucin domain-

containing-3 (Tim-3), lymphocyte activation gene 3 (LAG-3), and B- and T-lymphocyte attenuator (BTLA), these upregulations affect and limit ICI therapy and then limit the efficacy of immunotherapy (Emens et al., 2017). To remodelling the immunosuppressive TME and recruiting more new-born and healthy cytotoxic T cells to the tumour areas has become a crucial problem for developing cancer immunotherapy.

Therefore, immunotherapy based on tumour-associated macrophage reprogramming is developed and tries to address the issue of the lack of cytotoxicity T cells. This kind of immunotherapy differs from the ICI therapy and aims to reprogram TAMs to recruit more cytotoxic T cells to the solid tumour, resulting the cancer therapy.

5.3 A comparative analysis of CD-based formulations in PCa immunotherapy

Cyclodextrins (CDs) as non-viral vectors can be finely tuned to achieve the optimal or ideal properties for siRNA delivery, CDs are cyclic oligosaccharides derived from starch, consisting of at least 6 glucose subunits linked by α -1,4 glycosidic bonds, the primary and secondary sides of the ring structure are hydrophilic and the cavity is hydrophobic. The hydroxyl groups on the ring structure can be modified resulting in functionalised cyclodextrins suitable for different delivery requirements. Functionalised cyclodextrins, including cationic CDs and neutral PEGylated CDs, have been shown to be successful biomaterials for the delivery of therapeutic nucleic acids

including siRNA(Ortiz Mellet et al., 2010, Singh et al., 2019, O'Mahony et al., 2013, Haley et al., 2020).

In the thesis, PEGylated cationic CD vectors were designed to yield a positive charge and conjugated with ligands to lower the cytotoxicity and enhance transfection effects. Although the inclusion of PEG increased the size of the NPs, the size, approximately 250 nm, is still within the requirements of a nanoformulation. In addition, the overall positive charge of the siRNA.cationic CD complex was successfully decreased by the PEG chains resulting in formulations suitable for cell and animal use.

Table 5.1 provides a detailed comparison of the physicochemical properties and *in vivo/in vitro* results of the 2 nanoparticles designed in this thesis.

As previously described, CD169 is overexpressed on the surface of M2 macrophages with little or no expression on other cell types. Therefore, a sialic acid ligand based on the SA-CD169 axis was synthesised. Stearic acid-PEG-SA was synthesised from stearic acid-PEG-COOH and SA through esterification.

To enhance the therapeutic efficacy of the siRNA treatment, an M2pep-based ligand, DSPE-PEG-M2pep was synthesized via the Michael addition reaction using DSPE-PEG-Maleimide and M2pep. The particle size of M2pep-based siRNA NPs is approximately 250 nm, with a lower than 0.3 (0.25), which is considered acceptable for

nanocarrier drug delivery systems and indicates a homogenous population (Danaei et al., 2018). The surface charge is the lowest among all three NPs designed, at 11 mV (the two SA-targeted NPs present similar particle size and PDI, while the surface charge was higher, at 29 mV and 18 mV, respectively). Generally, the positive surface charge of NPs increases cellular uptake (Forest and Pourchez, 2017), however, the toxicity of the nanoparticles has been reported to increase with an increase in surface charge (Huang et al., 2017). Consequently, the lower charge associated with the M2pep.CD NPs is an advantage of this formulation.

The *in vitro* cell data showed that NPs with the M2pep ligand enhanced the cellular uptake in M2 macrophages (78.4% and 43.1% in RAW 264.7- and THP-1-derived M2 macrophages after 24 h transfection) and mediated a greater CSF-1R gene silencing (57.7% and 42.2% CSF-1R gene knockdown in RAW 264.7- and THP-1-derived M2 macrophages) than the SA-based NPs. While cellular uptake was observed using FACS and fluorescence microscopy, the degree to which endosomal escape influences the extent of gene knockdown requires further investigation. Simultaneously, 71.9% and 73.0% of RAW 264.7- and THP-1-derived M2 macrophages were reprogrammed to the M1 phenotype by the M2pep-based NPs, these values are higher than those observed with the SA-based NPs (Table. 5.1). Consequently, based on the lower surface charge and higher *in vitro* data the M2pep-targeted NP formulation was chosen for the *in vivo* study.

The M2pep-based NPs significantly reduced the tumour size (75.88%) and tumour weight (75.90%) in the PCa-bearing mouse model compared with the untreated group (Chapter 5). Similar tumour inhibition effects were observed in animal models of other cancers using the same ligand. Nanomicelles based on polyethylenimine-stearic acid with M2pep as the targeting ligand achieved ~73% decrease in tumour size when used to co-deliver CSF-1R siRNA and a PI3K- γ inhibitor, while nanomicelles containing CSF-1R siRNA alone caused ~50% tumour inhibition in a pancreatic cancer xenograft mouse models (Li et al., 2020).

A further study using NPs based on 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and cholesterol oleate (C.O) and conjugated with M2pep/ α -peptide-dual ligand containing CSF-1R siRNA inhibited approximately 87% of tumour volume down in the melanoma-bearing mouse model (Qian et al., 2017).

This study shows that targeted M2pep targeted CD-based NPs containing siRNA can be used to tackle PCa producing efficacy data similar to that reported for other cancer types as described above. In addition, this TAM-targeted immunotherapy approach produced stronger inhibition of PCa tumour growth relative to siRNA treatments targeting PCa cells alone. It has been reported previously that pH-triggered amphiphilic poly-l-lysine NPs containing VEGF siRNA reduced PCa growth by ~50% in the same tumour-bearing model (Guo et al., 2012a). In addition, a hepta-guanidino- β -cyclodextrin targeted with anisamide, a ligand for the sigma receptor overexpressed on

the surface of cancer cells, achieved ~65% reduction in PCa growth (Guo et al., 2012b).

In summary, this work indicates the potential of modified CDs to facilitate the targeted delivery of siRNA for immunotherapy of PCa. Scope exists to enhance the efficacy of these NPs by investigating the extent of endosomal escape and exploring methods to enhance it. In addition, the unique chemistry of the CDs presents the possibility of co-administration of traditional chemotherapy drugs via inclusion complex formation thus providing further opportunities to maximise the therapeutic outcome.

Table 5.1 Summary of CD NPs based on physical characteristics, *in vitro* and *in vivo* data.

	Chapter 2	Chapter 3 & 4
Targeting ligand	Stearic acid-PEG-sialic acid	DSPE-PEG-M2pep
Physical Characteristics		
Particle size (nm)	246 $\pm$ 16	253 $\pm$ 17
Zeta potential (mV)	29 $\pm$ 2	11 $\pm$ 3
PDI	0.27	0.25
Serum stability	Stable in 50% FBS for up to 24 h	Stable in 50% FBS for up to 24 h
Salt stability	No aggregation observed for up to 72 h in OptiMEM	No aggregation observed for up to 72 h in OptiMEM
<i>In Vitro</i> Characteristics		
Toxicity	No cytotoxic observed in THP-1- and RAW 264.7-derived M2 macrophages for up to 48 h with 100 nM of	No cytotoxic observed in THP-1- and RAW 264.7-derived M2 macrophages for up to 48 h with 100 nM of

	siRNA by CCK-8 assay	siRNA by CCK-8 assay
Cellular uptake in M2 macrophages	THP-1-derived: 17.9 % (6 h) & 37.6 % (24 h) RAW 264.7-derived: 49.0 % (6 h) & 63.8 % (24 h)	THP-1-derived: 19.5 % (6 h) & 43.1 % (24 h) RAW 264.7-derived: 52.0 % (6 h) & 78.4 % (24 h)
TAMs Reprogrammed & TME Remodelling (NPs containing CSF-1R siRNA alone)		
<i>CSF-1R</i> Gene Knockdown in M2 Macrophages (6 h transfection followed by 42 h more culture under normal condition)	29.6 % (THP-1-derived) & 38.9 % (RAW 264.7-derived)	42.2 % (THP-1-derived) & 57.7 % (RAW 264.7-derived)
TAMs Reprogrammed	THP-1-derived:	THP-1-derived:

Rates (6 h transfection followed by 42 h more culture under normal condition)	54.2 % (M1) & 44.4 % (M2) RAW 264.7-derived: 67.5 % (M1) & 35.9 % (M2)	73.0 % (M1) & 25.0 % (M2) RAW 264.7-derived: 71.9 % (M1) & 26.4 % (M2)
PCa Cells Apoptosis (%) (48 h transfection)	49.2 (PC-3) & 69.2 (TRAMP C1)	31.4 (PC-3) & 43.6 (TRAMP C1)
<i>In Vivo Data</i>		
Biodistribution	N/A	Up to 2.2-fold increase in tumour accumulation compared to non-targeted NPs
Toxicity	N/A	No observative toxicity in major organs by H & E staining
Tumour size	N/A	~ 2-fold decrease compared with non-targeted NPs

		~ 5-fold decrease compared with Untreated
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5.4 Future Perspectives

The studies in this thesis highlight the potential of cyclodextrin formulations for gene delivery in prostate cancer immunotherapy.

Despite the promising *in vivo* results achieved with our optimum prototype, M2pep NP, tumour volume continued to increase after the cessation of nanoparticle administration, a dilemma similar to that frequently seen with conventional treatment options. Consequently, the CD-based targeted siRNA NPs therapeutic, while promising, would benefit from further improvement. In addition, this treatment strategy needs to be tested in animal models which are more representative of the *in vivo* disease conditions such as orthotopic PCa models and metastatic tumour models.

In recent years, research has focused on methods to improve the efficacy of cancer immunotherapy including the use of a dual-targeting ligand to increase targeting efficiency (Qian et al., 2017); the blockages of two signalling pathways (combination of siRNA/antibody/inhibitor) (Ramesh et al., 2019, Kulkarni et al., 2018, Li et al., 2020); and a combination approach of blocking signalling pathways with anti-cancer drugs (Sun et al., 2022, Zou et al., 2021). Meanwhile, some combinations of siRNA and inhibitors or antibodies have also shown synergistic anti-tumour effects in the treatment of specific cancers (Gomez-Roca et al., 2022, Li et al., 2021), these concepts could be explored for future development of immunotherapy in prostate cancer. For example, a

combination approach based on the co-administration of current drugs used in PCa therapy, such as abiraterone or androgen receptor inhibitors for castrate-resistant prostate cancer, in combination with siRNA-based immunotherapy may be worthy of future investigation.

5.5 Goals Achieved

This thesis developed a range of amphiphilic cationic β -cyclodextrin-based nanoparticulate siRNAs delivery systems to deliver CSF-1R siRNA to M2 macrophages via sialic acid-CD169 or M2pep-M2 macrophage axes. The reprogrammed TAMs upregulated the expression of M1 cytokines/M1 chemokines, downregulated the levels of M2 cell factors, and caused both human and mouse prostate cancer cell apoptosis in a co-culture model of macrophages and cancer cells *in vitro*. Additionally, M2pep-based targeted CD nanoparticles were investigated in a TRAMP C1-bearing mouse model, resulting in reprogramming the phenotype of TAMs from M2 to M1, remodelling the immunosuppressive TME, recruiting cytotoxic T cells and T helper cells to the tumour area, and successfully limit the growth and development of prostate cancer. To date, this is the first report of this gene therapy-based TAM-targeted immunotherapy in the treatment of prostate cancer.

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