This dissertation explored various strategies for designing multivalent antibody recruiting molecules (ARMs) for use in cancer immunotherapy.

Chapter 1 introduces the functional mechanisms and composition of Antibody Recruiting Molecules (ARMs). ARMs are bifunctional molecules composed of an antibody-binding domain (ABD), typically a hapten for recruiting endogenous anti-hapten antibodies, and a target-binding domain (TBD), which is a target ligand for binding to the surface of tumor cells. ARMs mediate ternary complex formation between endogenous antibodies and cancer cells, thereby flagging cancer cells for destruction through innate immune defense mechanisms, including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC).

Over the past decades, significant advancements have been made in this field. In **Chapter 1**, various aspects of ARM development were reviewed, including the use of rhamnose, DNP, α-Gal, and FITC derivatives as ABDs, as well as the use of lipid chains, FA, uPAR, OPN, and PSMA as TBDs. Multiple studies have demonstrated successful antibody recruitment and immune-mediated killing of cancer cells both *in vivo* and *in vitro*. Furthermore, several studies have shown that multivalent ARMs offer advantages over monovalent ARMs by increasing the avidity of the ternary complex. Some ARMs have already entered clinical trials, and there is an outlook for more promising ARMs to undergo clinical evaluation in the future.

In Chapter 2, we focused on the synthesis and characterization of antibody-recruiting oligomers produced by solid-phase synthesis. Solid-phase synthesis is notable for its simplified purification process and high efficiency, which facilitates the parallel production of a large number of analogous compounds. The oligomeric ARM synthesis process began with a 2-chlorotrityl chloride polystyrene resin and involved conjugation with motifs via SN_2 reactions, followed by acylation with bromoacetic acid. In this work, alkyl lipids comprising 14 carbons, which randomly insert by hydrophobic interaction into the phospholipid bilayer of the plasma membrane, were used as TBD. DNP was used as ABD, and the length of the PEG spacer between the DNP motif and the oligomer backbone was evaluated for its influence on antibody recruiting efficiency *in vitro*.

Three series of oligomers were synthesized, each with varying numbers and positioning of ABDs and TBDs. From the first series of oligomers, one comprising two

ABD motifs featuring a PEG_2 spacer and two TBD motifs was identified as the most potent in antibody recruitment. Based on this structure, six more analogous oligomers were produced, expanding the number of units in the backbone. *In vitro* screening of these oligomers indicated solubility issues, likely due to the increased hydrophobicity of these structures. Therefore, a third series of oligomers was produced, in which we replaced the PEG2 spacer with a PEG_8 spacer. The resulting oligomers exhibited markedly improved solubility, and we identified a lead oligomer featuring a single ABD motif and two TBD motifs as the most potent antibody recruiter from all synthesized oligomers.

Subsequently, this oligomer was further conjugated with Rhodamine as a fluorescent dye and imidazoquinoline (IMDQ) as a TLR7/8 agonist to induce innate immune activation. The cellular interaction of this oligomer was assessed using flow cytometry and confocal microscopy, confirming effective cell binding and anti-DNP antibody recruitment to the cell surface. The activity of the IMDQ-conjugated oligomer was tested on RAW Blue cells, showing successful activation of TLR signaling *in vitro*. *In vivo*, in CT26 tumor-bearing IFN-$\beta^{(+/\Delta\beta)\text{-luc}}$ reporter mice, intratumoral injection of the tri-functional oligomer revealed innate immune activation in the tumor microenvironment, with concomitant localization of the Rhodamine-labeled oligomer within the tumor. Additionally, flow cytometry analysis of single-cell suspensions from dissected tumors confirmed the binding of the oligomer to the tumor cells and the capacity of the oligomer to recruit anti-DNP antibodies to cells in oligomer-injected tumors.

In Chapter 3, we focused on the synthesis of ARMs targeting carbonic anhydrase IX (CAIX), which is a cell surface protein that is over-expressed in hypoxic solid tumors. To this end, we selected an acetazolamide analogue as a TBD, owing to its high affinity for CAIX. DNP was used as ABD. Initially, monovalent ARMs, specifically AAZ-PEG2-DNP and AAZ-PEG_8-DNP with different lengths of PEG units as linkers, were synthesized and termed SL-ARM-1 and SL-ARM-2, respectively. When comparing the anti-DNP antibody recruitment capacities of these SL-ARMs, we found that SL-ARM-2, containing a longer PEG linker, outperformed SL-ARM-1 on SK-RC-52 cells, which overexpress CAIX on their surface. In contrast, N_3-PEG_2-DNP and N_3-PEG_8-DNP, which lack AAZ, showed no ability to recruit anti-DNP antibodies, confirming that the successful binding at the tumor cell surface was mediated by AAZ.

To explore the potential advantages of multivalency, dextran scaffolds with a molecular weight of 150 kDa were conjugated with multiple AAZ-PEG_8- N_3 and DNP-PEG_8-N_3 via CuAAC click chemistry, maintaining an AAZ/DNP ratio of 1:1. The comparison between SL-ARM-2 and dex-ARMs demonstrated the superior antibody recruitment capacity of multivalent ARMs over monovalent ARMs. In competition experiments, free AAZ was shown to inhibit the binding of ARMs to the tumor cell surface in a concentration-dependent manner, highlighting the specific binding of AAZ to the CAIX target ligand. Different ratios of AAZ and DNP (1:1, 3:1, and 1:3) were conjugated to dextran, showing concentration-dependent antibody recruitment to the tumor cell surface. However, the differences in ligand ratio had a minor impact, indicating the good robustness of the dex-ARMs system. Additionally, mice were immunized with KLH-DNP adjuvanted with LNP (poly(I:C)), and their serum was collected. Anti-DNP antibodies were quantified from the serum via ELISA. Both SL-ARM-2 and dex-ARMs successfully recruited IgG1 antibodies from the serum to SK-RC-52 cells. The distinct performance of both ARMs in binding IgG1 highlighted the advantage of multivalency, which is consistent with the research described in this study and the published work reviewed in **Chapter 1**.

In Chapter 4, pH-sensitive polymer- and block polymer-based ARMs were synthesized to exploit the acidic pH of solid tumors, which results from high metabolic rates and lactic acid production from tumor cells, as a trigger for the recruitment of endogenous antibodies to cell surfaces. The polymers were synthesized by RAFT polymerization of pentafluorophenyl methacrylate (PFPMA). These polymers were then functionalized with DNP motifs as ABDs, and alkyl lipid chains and ionizable azepanyl motifs as TBDs. The antibody recruitment capacity of these polymers was tested at both neutral and acidic pH levels. Notably, the inclusion of ionizable azepanyl improved the binding capacity of the polymers to tumor cell surfaces under acidic conditions, demonstrating enhanced antibody recruitment in a tumor-mimicking acidic environment.

To further exploit the pH-sensitive properties of the azepanyl motifs, an amphiphilic block copolymer was synthesized, composed of methoxytriethylene glycol methacrylate (MEO3MA) as a hydrophilic block and PFPMA as a scaffold for the subsequent conjugation of DNP and azepanyl motifs. The resulting block copolymer could form micelles in an aqueous buffer at neutral pH, whereas at an acidic pH of 5.5,

the micelles disassembled into soluble unimers. The zeta potential of the micelles significantly decreased from pH 5 to 9, confirming their pH-dependent behavior. As a control, a block polymer decorated only with azepanyl amine (without DNP) exhibited similar particle characteristics. The capacity of these block polymers to bind anti-DNP antibodies and their micelle binding efficiency were evaluated on the CT26 tumor cell line. The results showed higher micelle binding and antibody recruitment at pH 6 compared to neutral pH.

In **Chapter 5**, the broader international context of this dissertation was described, highlighting the current state and challenges of tumor therapy. Immunotherapy plays a crucial role in cancer treatment, with numerous commercially available medicines and strategies such as monoclonal antibodies (mAbs) and chimeric antigen receptor T (CAR-T) cells. Instead of blocking the activity of specific cell surface proteins or inducing apoptosis, several mAbs also aim to activate the innate immune response, including ADCC, ADCP, and CDC. Similarly, ARMs facilitate tumor cell killing through innate immune responses based on endogenous antibodies, offering low toxicity compared to mAbs, potentially making them a good alternative.

ARMs are part of a broader category of bifunctional molecules developed for immunotherapy. In recent years, various bifunctional molecules have been proposed in the field of immunotherapy. Notably, targeted protein degradation (TPD) chimeras, including proteolysis-targeting chimeras (PROTACs), autophagy-targeting chimeras (AUTACs), autophagosome-tethering compounds (ATTECs), lysosome-targeting chimeras (LYTACs), and antibody-based PROTACs (AbTACs), have been designed to degrade specific proteins of interest (POIs). By targeting and degrading tumor-related POIs, these chimeras offer a novel approach to killing tumor cells. Additionally, antibody-drug conjugates (ADCs), which directly induce cytotoxicity in tumor cells, have shown promising efficacy in cancer treatment. Several ADCs have already been approved for clinical use. The ongoing development of bifunctional molecules and their incorporation into clinical practice highlight the dynamic and evolving landscape of cancer immunotherapy, aiming to improve treatment outcomes and reduce side effects.