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Title Page

Impact of CEA-targeting Nanoparticles for Drug Delivery in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of the most common cause of cancer-related death in the world, mainly due to distant metastasis events. Developing targeted strategies to treat and follow individuals in more developed stages is needed. The carcinoembryonic antigen (CEA) is a cell surface-overexpressed glycoprotein in most CRC patients and the evaluation of its serum levels is recommended in the clinics. These reasons motivated the production of CEA-targeted nanotechnologies for monitorization of CRC progression, but only a few reports its usage for drug delivery. The cellular internalization of CEA-linked nanosystems occurs by the natural recycling of the CEA itself, enabling its longer retention and sustained release of the cargo. The functionalization of nanoparticles with less affinity ligands for CEA is possibly the best choice to avoid its binding to the soluble CEA. Here, we underline also the usage of nanoparticles made of poly(lactic-*co*-glycolic acid) (PLGA) polymer, a well-known material, due to its biocompatibility and low toxicity properties. This work will preferentially refer the contributions of antibody fragment-functionalized nanoparticles, as promising high affinity molecules to decorate nanosystems. The linkers and conjugation chemistries chosen for ligand-nanoparticle coupling will be herein addressed as essential to modulate nanosystems features. This review, from our knowledge, is the first that focus on CEA-targeted nanotechnologies to serve colorectal cancer therapy and monitorization.

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Introduction

Colorectal cancer (CRC) is the 3rd more incident, the fourth most common cause of cancer-related death and the third with the highest 5-year prevalence (post treatment) in the world (Organization, 2012). This type of malignant neoplasm arises from the mucosa of the colon or the rectum and could follow one of the three mechanisms of tumorigenesis, or a combination of them, categorized as chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP). The chromosomal instability represents the pathway that most of sporadic colorectal malignant neoplasms follow (Kotelevets et al., 2016; Tariq and Ghias, 2016).

The major reason for treatment failure in CRC is the development of distant metastasis, more commonly liver metastasis. The production of technologies that specifically target CRC cells in more developed stages of their tumorigenesis is possibly a good effort to overcome the collateral damages caused by 'blind therapies' as standard chemotherapeutics. The targeted nanocarriers for drug delivery (Dinarvand et al., 2011), which specifically recognize cell-surface overexpressed molecules, are already a field of interest. In this review, we investigated several promising molecules and focused on the potential of carcinoembryonic antigen (CEA), that is considered the most expressed protein in CRC (James P Tiernan, 2015), as a targeting moiety to direct the formulation, either for simple disease monitorization or targeted drug delivery purposes.

In the context of targeted nanotechnology, we will preferably approach the functionalization of nanoparticles with antibody fragments, as they conserve the high affinity characteristics of a monoclonal antibody with more potential for oriented functionalization (Cheng and Allen, 2010; Vahid Heravi Shargh, 2016). A summary about the antibody features will be given to complete the logical progression of the work.

Here, we also defend the functionalization of nanoparticles made of poly(lactic-*co*-glycolic acid) (PLGA) polymers, being some of them FDA-approved materials with huge impact, due to its biocompatibility and low toxicity features (Murthy, 2007). The most common antibody-conjugation strategies will be addressed as some most suited linkers currently used, as they are important in the modulation of the nanosystem properties.

We believe that in the near future CEA targeting nanotechnologies might offer novel and more efficient anticancer theragnostic strategies.

Cell surface molecules highly expressed on CRC

Targeted technologies to diagnose, to evaluate the prognostic or the predictive response to a treatment, and even to treat tumours rely on identifying molecular entities characteristic, or at least, highly expressed in neoplastic rather than normal tissues. The histological features and genetic signature of certain tumours permit the stratification into distinct subtypes, providing in some cases a reliable prediction of response to a targeted therapy (Tiernan et al., 2013; Freidlin and Korn, 2014).

One of the most consensual definitions of tumour marker is given by the National Cancer Institute (NCI), from the National Institutes of Health (NIH), as entities, most of them proteins that are produced by cancerous or noncancerous cells in response to malign or benign events. When referring to a malignancy, they exist in higher levels and can be found in tissues or body fluids of some cancer patients (Institute).

One factor that cannot be discard is that the presence of a certain tumour marker in a patient is not always correspondent to a predicted clinical state or response to a treatment, and sometimes the variation between measurements into a population could be high, which invalidates its usage (Strimbu and Tavel, 2010).

The nanosystems made to specifically deliver a diagnostic probe or a therapeutic agent to the inside of a cancerous cell, require to be firstly highly targeted to a cell-surface molecule, and ideally, specifically expressed in the malign phenotype of study. For this reason, it is necessary to understand which are the molecular options that remain available for the targeting of colorectal cancer cells. The most common overexpressed cell surface molecules in colorectal cancer are the cell-adhesion protein carcinoembryonic antigen (CEA), the tumour-associated glycoprotein-72 (TAG-72), the folate receptor – α (FRα) and the epithelial growth factor receptor (EGFR), that are present at 98.8 %, 79.0 %, 37.1 % and 32.8 % of cases, respectively, when compared with matched health tissues (Tiernan et al., 2013). Another study suggests that CD44v6 overexpression, a hyaluronic acid (HA) receptor, represents a poor prognostic factor for colorectal adenocarcinoma patients (Kobel et al., 2004). Other authors confirmed the existence of a higher level of serum carbohydrate antigen 19-9 (CA 19-9) and of alpha fetoprotein (AFP) in colorectal cancer patients, rather than in patients with non-malignant colorectal disease (NMCD) (Wang et al., 2014). In addition, the vascular endothelial growth factor receptor (VEGFR) and the transferrin receptor protein 1 (TfR1) are also upregulated in CRC (Hasan et al., 2011; Miljus et al., 2015). Other relevant cell surface molecule is tyrosine kinase receptor c-MET, that is highly expressed in colorectal cancer and in liver metastases of this malignant neoplasm (Conor A. Bradley, 2016). Lastly, the

death receptor 5 (DR-5) is a cell-surface receptor with pro-apoptotic characteristics that is overexpressed in stage II and III colorectal cancer patients (Schmid et al., 2014). Table 1 represent some of nanoparticle-based targeting systems to current most promising cell-surface molecules for gastrointestinal cancer treatment and monitorization.

In clinics, the tumour biomarkers that are also cell-surface molecules, currently used either for disease monitoring, diagnostic, prognostic and predictive response in colorectal cancer are few. The CEA is indicated for several situations: i) stage II patients' prognosis, ii) preoperative evaluation of newly diagnosed cases, iii) postoperative surveillance and iv) in advanced disease monitorization. The CA 19-9 (a cell-surface carbohydrate antigen) has emerged, although not yet FDA-recommended, as a postoperative surveillance marker, in cases of metastatic disease, when CEA is not upregulated (Duffy et al., 2014). The overexpression of MET and of human epidermal growth factor receptor 2 (HER2) configures *de novo* resistance to anti-EGFR immunotherapy (HER3 and EGFR mutations were not clearly associated). Despite of this, the overexpression evaluation of EGFR, HER2, MET or HER3 are not recommended for CRC patients (Van Cutsem et al., 2016). In ultimate analysis, CEA is an overexpressed protein in the most CRC cases, and the only cell-surface molecule recommended for colorectal cancer patients' management. These reasons motivate the selection of CEA as a promising molecule for nanoparticle-targeting systems in colorectal cancer.

Carcinoembryonic antigen as a target for CRC-directed therapies

CEA features

Carcinoembryonic antigen (CEA) is a glycoprotein that belongs to the 12 members-family of carcinoembryonic antigen cell adhesion molecules (CEACAM), as represented on Figure 1. On its turn, CEACAMs belong to the superfamily of immunoglobulins (Igs) and are generally characterized by harbouring one variable (IgV-like) N-terminal domain, homologous to the Ig variable domain, responsible for the binding to homophilic and heterophilic cell adhesion molecules. This terminal N-domain is generally linked to none or a maximum of 6 constant domains (IgC2-like), also homologous to immunoglobulins non-variable domains. In the specific case of the CEA protein, also known as CEACAM5 or CD66e, once produced it is covalently bound to glycosylphosphatidylinositol (GPI), and this post-translational modification leads to the anchorage of CEA at the external

surface of the phospholipidic bilayer. This GPI-anchorage to the membrane does not allow CEA to perform by itself any transduction of signal since it lacks intracellular domains, requiring transactivation through other intracellular partners (Maeda and Kinoshita, 2011; Beauchemin and Arabzadeh, 2013).

Carcinoembryonic antigen (CEA) is produced in human gastrointestinal tract during early stages of embryonic and foetal development (from 9 to 14 weeks), and before birth its serum levels decrease, remaining very low in the adult life (Rodrigues et al., 2018). Nevertheless, there are some structures that still produce CEA afterwards. Its expression is mainly observed in goblet and columnar epithelial cells of the colon, namely in the free luminal surface and at the upper third of the crypt. It is also present in prostate, cervix, tongue, esophagus, stomach and sweet glands (Hammarstrom, 1999). An healthy adult produce about 50-70 mg/day of CEA from the apical surface of mature enterocytes and release it extracellularly into the gut lumen, which will get to the exterior environment with the defecation process (Hammarstrom, 1999; Ruckert et al., 2010).

When referring to glycoproteins, the linkage between the polypeptide backbone and the glycans typically occurs through two chemical strategies: i) the binding of the nitrogen atom of an asparagine residue to a glycan chain (N-glycans), as the case of CEACAM5; ii) or the binding of an oxygen atom of a serine or threonine residue to a glycan chain (O-glycans), like mucins. Glycoproteins as CEA, either in normal or neoplastic forms, are highly N-linked to oligosaccharides (Reis et al., 2010). Namely, colorectal neoplasms produce high levels of CEA glycosylated forms that can reach the blood vessels, and at this point, can be detected into circulation. Indeed, in practice, the molecular mass of CEA is 180-200 kDa, and about 60% of this value is due to N-glycosylation. However, the theoretical molecular weight of the full-length protein, after deglycosylation treatment, decreases approximately to 80 kDa. Notably, the glycosylated patterns of CEA differ amongst tissues and cells. There are described isoforms, being the most abundant the splice variant derived from isoform 5D that has 60 kDa; and the splice variant derived from isoform 3D with estimated 40 kDa (Hatakeyama et al., 2013).

Importantly, CEA protein expression is associated with melanoma, lung adenocarcinoma, mucinous ovarian carcinoma, and it is mostly seen in digestive tract cancers as pancreatic, gastric and namely, colorectal carcinomas (Hammarstrom, 1999; Beauchemin and Arabzadeh, 2013). In opposition to an healthy context, in which colon cells express CEA only through the apical side, once the tumorigenic process occurs, there is no more defined basal lamina in the tissue, cells lose polarity and CEA is

expressed in the entire surface (Hammarstrom, 1999). The CEA importance in oncology, namely in colorectal cancer, is highlighted by multiple clinical trials in Table 2.

Recycling of the CEA protein

This oncofoetal molecule is more often referred as a non-internalizing antigen. Besides, Jeffrey Bryan *et al*. studied the internalization and biodistribution of CEA at several time points. To achieve this, they used two antibodies, an anti-CEA mAb and a known rapid internalized monoclonal antibody, both labelled with a radionucleotide (cooper-64). They tested labelled mAbs in mouse xenografts from LS174T colorectal cancer cells. The results revealed that CEA had a fast blood clearance, an increased liver uptake and enhanced tumour vascular accumulation when compared with the supposed fast internalized antibody. These events suggested that CEA is continuously secreted by the tumour to the bloodstream and right after is cleared by receptor-mediated endocytosis in the hepatic cells. The secreted CEA, as the authors suggested, is probably immediately coupled to the CEA targeted mAb, establishing CEA-antibody complexes that could explain the fast appearance of radioactivity in the liver. Besides, the own affinity of the antibody influences its cellular penetration, being the high affinity ones more susceptible of binding firstly to the soluble CEA, leaving only a few to bind to the membrane-linked CEA, decreasing in this way the antibody penetration within tumours (Bryan et al., 2005).

Once inside the body, an antibody is immediately exposed to the bloodstream and clearance, extravasation from capillary vessels, tumour diffusion, internalization and finally, catabolic degradation in cancer cells (Jain, 2001). Another recent line of thinking is suggested by K. Dane Wittrup *et al*. that compared the CEA detection using different antibodies and namely the internalization rate constant (K_e) of an mAb anti-CEA and two single-chain variable fragments (scFvs) anti-CEA, the Sm3E (Vigor et al., 2010) and shMFE (Schumacher et al., 2013), latter referred in this dissertation, in several CRC cell lines (Schmidt et al., 2008). The team interest on evaluating different antibodies is also due to their potential to transport pharma, either only using an antibody associated-drug or an antibody tagged-nanosystem carrying the drug. One factor that is certainly delaying the success of antibody technologies for drug deliver is precisely its lack of penetration in cancer cells (Vahid Heravi Shargh, 2016). The cellular internalization followed by antibody-ligand binding, and consequent catabolism that occurs inside the cell, decrease the penetration ability of the antibody, and by its turn, the penetration of the drug associated (Schmidt et al., 2008). The monoclonal antibody tested, independently of its own affinity, exhibited a similar slow uptake by CRC cells (10-16h), compatible with the

metabolic turnover of the CEA protein (~15 h). The uptake was enough to guarantee its distribution and retention in the cells. Importantly, none of the antibodies tested triggered changes in CEA expression. The hypothesis that is given by K. Dane Wittrup's team, infers that the uptake of the antibodies into CRC cells results from a non-specific signalling mechanism and from the natural recycling of the CEA itself. In this way, it often underlines the role of CEA as a GPI-linked protein, with no known ability to trigger signalling transduction pathways. Antibodies with slower internalization rates, as surface molecules with slow turnovers, are more likely to enhance the penetration and retention in the tumour cells (Schmidt et al., 2008). Once the internalization into a CRC cell occurs by non-specific mechanisms, the usage of less affinity ligands for CEA recognition is probably the best choice when the main objective is the sustained intravenous release of drugs, avoiding thereby its binding to soluble CEA.

Nanoparticles: An opportunity for safe drug delivery

Drug delivery systems have been developed to improve the transport of therapeutic entities through the biological fluids of the body, enhancing their half-life time in circulation, and decreasing their side effects, namely toxicity (Robert et al., 2017). The major role of drug delivery strategies not only comprises the overcoming of poor solubility and stability of standard therapies, giving the opportunity to test known drugs that otherwise would be ignored; but could even be applied to novel therapeutic entities, giving them the ability of overcoming biological barriers and making them more specific for tumour cells (Allen, 2002; Ferrari, 2005).

The promising contributions of such technologies has attracted the attention of cancer researchers and physicians around the world. The chemotherapy, radiotherapy and surgery resection remain as the three "gold standards" anti-cancer therapies. Nevertheless, the majority of the standard chemotherapies approved for the clinical usage have no ability to distinguish normal from cancer cells. This leads to severe side effects, namely in fast-growing cells, once those drugs act generally in impairing mitosis. Those cells include hair follicles, cells from bone marrow and gastrointestinal system, leading to hair loss, immune system failure and infections, respectively (Banerjee and Sengupta, 2011; Labianca et al., 2013; Steichen et al., 2013).

Drug nanocarriers are solid and colloidal particles that emerge as safe drug vehicles, designed to generate much fewer toxic side effects and deliver high quantities of cargo to a very specific site of interest (Richards et al., 2017). Nanoparticles allow 1 – 1000 nm

diameter (Azevedo et al., 2018), however being the < 200 nm ones the most suited for intravenous administrations, considering the width of body microcapillaries. Their advantages over microparticles (with a diameter > 1 μm) are notable, once the diameter of the body capillaries are 5-6 μm and particles over 5 μm could aggregate and drive an embolism (Singh and Lillard, 2009).

The novel therapies produced so far that are currently used for colorectal cancer, include targeted agents, as monoclonal antibodies anti-VEGF like Bevacizumab (de Gramont et al., 2012), or anti-EGFR as Cetuximab (Alberts SR, 2012) and Panitumumab, the anti-VEGF recombinant fusion protein Aflibercept, and the multikinase inhibitor Regorafenib (Van Cutsem et al., 2014). For early colorectal cancer, no biological targeted drugs are actually recommended (Labianca et al., 2013). Additionally, for metastatic CRC conditions, the majority of these therapies, namely the monoclonal antibodies, only evidence clinical benefit when combined with standard chemotherapeutics (Van Cutsem et al., 2014; Van Cutsem et al., 2016). Most of the work that have been done on encapsulating those novel targeted molecules, like monoclonal antibodies, only intended the encapsulation of a single drug. Nevertheless, as most of them are only useful when combined with standard therapies, it is perhaps more interesting to encapsulate the whole combinatorial therapeutic scheme into the particles, instead of just an entity of it.

When developing a new formulation for therapeutic purposes, there are main objectives to accomplish. Firstly, to guarantee that the system is biocompatible and stable in body fluids, which can be ensured by properly coating the particle surface with materials, as poly(ethylene glycol) PEG, that avoid the adhesion of opsonins, permitting to escape to the immune system surveillance. Secondly, to increase the concentration of drug into the tumour tissue, by using materials that increase the tumour enhanced permeation and retention (EPR) effect, or simply by targeting the whole system to a molecule highly expressed in the tumour but not in healthy tissues. Finally, to reduce the toxic side effects of the drug, either by simply encapsulation, or encapsulating the drug within a targeted system (Dawidczyk et al., 2014).

In the field of targeted drug delivery, strategies can be sorted through passive or active targeting. The targeted system, as other non-targeted vehicles, will be into the bloodstream. The difference is that the term ‘passive targeting’ is used as a synonymous of “blood circulation and extravasation”, meaning the passive accumulation of drugs in the vasculature surrounding the tumour, followed by an extravasation to tumour tissues, where it will be distributed (Park, 2013). The active targeting happens only after the “blood circulation and extravasation” where it occurs a specific interaction with a ligand

from the drug/vehicle and a certain cancer cell molecule. The nanoparticles' surface can also be functionalized with molecules that have affinity to a specific cellular target of cancer cells as surface receptors and soluble proteins, to direct the whole system to a specific site (Zalba et al., 2015).

One characteristic that tumours have, although not exclusively, that might increase nanoparticles passive or active recruitment, is the enhanced permeation and retention effect, known as EPR effect. The EPR effect is a phenomenon observed for macromolecules such as certain proteins and polymers with a molecular weight higher than 40-50 kDa. Such effect favours molecules and nanoparticles delivery systems preferential accumulation in the neoplastic tissue rather than in healthy tissue, increasing the local concentration of a given drug (Hongzhuan Yin, 2014). The main reason for this behaviour is the defective hypervascularization with lacking of lymphatic drainage of the damaged tissues, so these molecules can invade the tumour tissue without being cleared for long time (Yin H, 2014). The inherent properties associated to these specific materials make them suitable to use in pharmaceutical formulations to enhance the accumulation of a drug into a solid neoplasm.

Nanocarriers can be sorted into organic (liposomes, polymeric micelles, polymeric nanoparticles and dendrimers), inorganic (iron oxide nanoparticles, gold nanoparticles, mesoporous silica nanoparticles, carbon nanoparticles and quantum dots), and hybrid organic-inorganic particles (Richards et al., 2017). One polymer that has become a success regarding polymeric nanoparticles is the poly(lactic-*co*-glycolic acid) (PLGA), mainly due to its biodegradability and low cellular toxicity (Murthy, 2007). Some PLGA polymers are FDA-approved materials and until nowadays several formulations of PLGA nanoparticles were clinically introduced, namely for advanced prostate cancer, ELIGARD®, that delivers leuprolide, the luteinizing hormone-releasing hormone (LHRH) that inhibits testosterone expression (Berges, 2005). Importantly, PLGA nanoparticles are versatile systems since, depending on the production method, can deliver hydrophobic (Le Broc-Ryckewaert et al., 2013) or hydrophilic drugs (Gomes et al., 2017). The functionalization of this polymer with poly(ethylene glycol) (PEG) turns the system less immunogenic, difficult its internalization and subsequent degradation by cancer cells, enhances its stability in the body and its accumulation on solid tumours, profiting from the described EPR effect (Oliveira et al., 2012; Dawidczyk et al., 2014). For the reasons above mentioned, PLGA polymeric nanoparticles will be privileged here.

CEA-targeting nanotechnologies

To create a targeted nanoparticle, is necessary to tag at its surface a molecule that will specifically bind to a cell-surface receptor characteristic of a pathology, or at least overexpressed in comparison to normal tissues, or even any extracellular molecule of interest. The functionalization of nanoparticles with specific ligands is currently a field of development, and several types of molecules can be used, considering the desired application. The ligands explored until nowadays include vitamins (Mallakpour and Soltanian, 2016), proteins (Wang et al., 2010), peptides (Lijun Ma, 2017), aptamers (Yang et al., 2015), monoclonal antibodies (Heister et al., 2009), and antibody fragments (Che-Ming Jack Hu, 2010). The last one covers a variety of entities as: i) $F(ab)'_2$, Fab', Fab and half-antibodies - hAb (~67kDa), native antibody fragments (Figure 2B), which can be produced by introducing specific enzymes or chemicals to cleave strategic points of a total immunoglobulin (Kennedy et al., 2017b); and ii) single-chain variable fragments - scFv (~27 kDa), single-domain antibody fragments - sdAb (~13 kDa) and SS-Fc bispecific fragments (~80 kDa), genetically-engineered antibody fragments (Figure 2C), generally produced by recombinant technologies like phage display techniques (Kennedy et al., 2017b).

The high affinity properties found in antibodies led to multiple applications in medicine, as the emerging immunotherapy. Nowadays, antibody fragments are arising as a new and improved technology that relies on full-antibody features with more advantages for conjugation to nanoparticles and tissue penetration (Richards et al., 2017).

Most of the applications of anti-CEA nanomaterials are used for detection of the secreted CEA protein itself, or even in the detection of CEA-overexpressing cells such as colorectal or pancreatic cancer cells (Vigor et al., 2010; Ramos-Gomes et al., 2018). Despite of the huge potential of new tools to detect CEA as monitoring purposes, only a few are working in CEA-targeting systems to enhance the efficiency of cancer therapy in more developed stages (Heister et al., 2009; Che-Ming Jack Hu, 2010). The Table 3 focus on the CEA-targeted nanotechnologies that can be applied to colorectal cancer therapy and monitorization. From now on, this dissertation will preferentially refer the contributions of antibodies, more specifically antibody fragments as promising molecules to nanoparticles driven therapies.

Active targeting moieties

Aptamers

Aptamers are usually non-immunogenic, single-stranded, synthetic oligonucleotides from RNA or DNA that can bind specifically to cell surface molecules. The small size of aptamers (from 20 to 50 nucleotides) allow them to work as deliver vehicles into the intracellular space. Although not able to passively permeate through biological membranes, these molecules overcome the phospholipidic bilayer through specific binding to cellular receptors that have turnover metabolisms compatible with the degradation time of the aptamer. Ultimately, they exhibit nano to picomolar affinities for their targets (Orava et al., 2010; Yang et al., 2015; Li et al., 2016a).

Monoclonal antibodies

The soluble form of antibodies is produced by professional B lymphocytes (plasmocytes), and there exist several manners of artificially fabricate antibodies against a desired protein epitope of an antigen. Each B lymphocyte clone produces antibodies that are specific for only a single epitope. A monoclonal antibody is in this way, an antibody produced by a single clone of B cells. To produce monoclonal antibodies of interest, host animals are first immunized with a specific immunogenic sequence of a given antigen, the epitope. Once immature B cells, non-reactive to host-antigens, migrate to the host spleen, they follow the maturation step where they are presented to the foreign antigen previously introduced. Still in the spleen, mature B lymphocytes, expressing at their surface the Ig receptors recognizing specifically the desired epitope, are selected and isolated. Those B cells are then fused with immortal B cancer cells, the myeloma cells, to constitute a highly proliferative hybridoma, immortal producers of that monoclonal antibody (Tsumoto, 2011).

As exposed in Figure 2A, each full length-immunoglobulin (~150 kDa) is composed by two Heavy chains (H, in blue) and two Light chains (L, in green). Within each chain there are two separated regions, the amino-terminal Variable region (V), containing VH and VL domains, and the carboxyl-terminal Constant region (C), containing CH1, CH2 and CH3 domains. Disulphide bridges are essential to link all chains and create the 'Y' shape characteristic of an Ig. In addition, each heavy (VH) or light variable (VL) region contains a hypervariable domain, composed by three protein loops, the Complementary-

Determining Regions (CDRs). The CDRs have different amino acid sequences from antibody to antibody, which make them responsible for the variety of antigen epitopes that antibodies can specifically recognize (Kennedy et al., 2017b).

Moreover, the full antibody has two fragment antigen binding (Fab) regions that integrate the sites for antigen binding (hypervariable regions) and the constant regions from heavy (CH1) and light (CL) chains. The Fragment crystallisable (Fc) region is the antibody portion that activates cells containing Fc receptors (FcR), namely phagocytic cells. Phagocytes have in this way the ability of trigger an immunological response through antibody-dependent cell-mediated cytotoxicity (ADCC). Fc fragments also initiate complement activation through the classical pathway, which ends with cell lysis (Kennedy et al., 2017a). Interestingly, immunoglobulins and albumin are the most abundant proteins present in human serum. To not waste much energy by producing *de novo* these proteins, the body has specific mechanisms to prolong their half-life in circulation. Particularly, FcRn (neonatal Fc Receptor) appears as an intracellular Fc-receptor that recognizes antibodies Fc domains and albumin, avoiding their degradation by lysosomes, which is an advantage of using whole Ig for targeting proposes (Martins et al., 2016).

Antibody fragments

Some drawbacks of whole antibodies are the immunogenicity and the clearance from bloodstream, both due to binding of Fc receptor-containing entities to antibody Fc region, (Cheng and Allen, 2010). In addition, antibody size (~150kDa) difficult cell penetration. Besides this, the bigger advantage of using a full-length mAb for targeting systems is the presence of two antigen binding regions (Fab), while some antibody fragments carry only one.

Antibody fragments, excluding SS-Fc ones (Li et al., 2016b), have multiple advantages in comparison to mAb, regarding their use in intracellular drug delivery systems. Firstly, they are less immunogenic than a whole Ig due to the lack of the Fc region, retaining almost the affinity and specificity found in whole immunoglobulins, and secondly, they are able to couple in a more oriented manner to a nanoparticulate system (Cheng and Allen, 2010; Vahid Heravi Shargh, 2016). For nanoparticle-decorating purposes, the size of the ligands is also important, making antibody fragments a certainly very promising toll.

Antibody conjugation strategies

To covalently link two compounds, it is first necessary to understand the reactive groups that are present in each of them. Next, it is required to choose the most appropriate crosslinker to participate in the selected conjugation reaction. When referring to antibody conjugation systems, there are two main chemistries that might be applied: the carbodiimide and the maleimide one. Importantly, the conjugation chemistry that is selected to bind a antibody to a nanoparticle can influence the specific binding to a desired epitope (James P Tiernan, 2015). As explored below, the linker chosen for ligand-nanoparticle coupling is essential to modulate the nanosystem characteristics.

Carboxyl-to-Amine conjugation reaction

This strategy is many times applied to covalently link the amine-containing residues (lysine, histidine and arginine) to a carboxylated structure or carboxyl-containing residues, as aspartic acid and glutamic acid, to a primary amine structure.

The first of a two-step reaction of carbodiimide chemical conjugation, where 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), a carbodiimide linker, reacts with the carboxylated structure is represented in Figure 3.The production of a relatively more stable and water-soluble ester complex is achieved through the addition of sulfo-NHS, *N*-hydroxysulfosuccinimide, representing the second step of the reaction. Thereby, the carboxyl-activated groups of the structure react with the primary amine groups of the antibody fragment (scFv), producing ultimately a stable amide between both. More importantly, the carbodiimide is known as a “zero-length” linker, meaning that the unstable intermediate *o*-Acylisourea will not participate in the final product of the reaction. The same happens when carbodiimide is used in combination with NHS or sulfo-NHS (NHS linked to a sulfonate group – SO_3^-) (Thomas Carter, 2016). Interestingly, James P Tiernan and collaborators tested two different linkers to conjugate a monoclonal antibody to nanoparticles by the carbodiimide chemistry: the EDC/NHS and the polyamidoamine (PAMAM) dendrimers. These dendrimers have primary amine groups at their surface that could either bound to the carboxylated silica nanoparticles or to the antibody. Authors studied the specificity of the conjugated systems, by conjugating separately with a negative control monoclonal antibody. Overall, they demonstrated that the EDC/NHS linkers provided 1.7-fold more binding comparing with the negative control, although not sufficient to guarantee specific binding. Moreover, the PAMAM dendrimers linked via carbodiimide chemistry showed a maximum binding of 12.3-fold comparing with negative control. These results could be explained due to the amplification of the conjugation

when using crosslinkers that bind to multiple molecules. In this case, each PAMAM dendrimer binds a single nanoparticle to several antibodies, amplifying the number of ligands that exist in the system, and therefore, increasing the available ligand epitopes for CEA receptor targeting. The article also alerts for the importance of using negative control antibodies to confirm that the binding of an antibody-functionalized nanoparticle is only due to the affinity of the antibody to its target epitope, and not due to nonspecific interactions that may occur (James P Tiernan, 2015).

Maleimide-to-sulfhydryl conjugation reaction

This chemical reaction is mostly applied to conjugate antibodies containing cysteine residues, that is the only amino acid containing a terminal thiol group. Such ligands can have just one sulfhydryl group (-SH) or multiple cysteines, which originates disulphide bridges (-S-S). The free cysteine amino acid is known as a relative rare constituent of proteins, and this feature is used as an advantage to artificially modify antibodies and another proteins of interest to produce the chemical conjugations desired through sulfhydryl binding reagents (Jones et al., 2012). A structure containing the maleimide group (crosslinker) could then react with the previously reduced thiol groups of the scFv antibody fragment, as exposed in Figure 3B. The final product would be a stable thioether linkage between both compounds. Regarding the maleimide molecule, the 'X' groups on it (Figure 3B) will not participate in the final product of the reaction. Such groups are generally any hydrogen atom (-H), and preferably any good-leaving group as the bromine atom (-Br) and other halogens, for instance. Once the maleimide reaction is known as an irreversible one, it could change the conformation of the antibodies, which could affect its affinity to the target. Baker and colleagues used halogen-substituted maleimides as dibromomaleimides, that have the ability to create a rigid two-carbon bridge between two cysteines (not represented). This strategy confers a reversible linkage and maintains the stability of the antibody (Schumacher et al., 2011; Schumacher et al., 2013). Moreover, James P Tiernan and co-workers tested two different crosslinkers: succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) and (succinimidyl-[(N-maleimidopropionamido)-tetraethyleneglycol] ester), (SM[PEG]$_4$), with the main goal of linking the amine groups previously added to the silica nanoparticles to the thiol groups of a monoclonal antibody (James P Tiernan, 2015). First the linkers reacted with the amine-coated silica particles and after this, the antibodies were added. Both crosslinkers have a NHS and a maleimide terminal group, one at each side, that will trigger, respectively, the binding of the amine groups of silica particles to the carboxyl-activated

linker, and the binding of the antibody thiol groups to the maleimide molecule. Both linkers did not show any specific binding to the neoplastic cells.

Conclusions

Colorectal cancer is one of the deadliest diseases worldwide, namely due to metastatic events. Developing new targeted strategies diagnose, treat and monitor individuals in less advanced stages is needed.

CEA glycoprotein appears as a cell surface molecule overexpressed in most CRC patients and the evaluation of its serum levels are recommended in the clinics. This promising protein has a slow turnover half-life (~15h), which enables the longer retention of ligand-CEA complexes inside the cell. By its turn, this could enhance the sustaining release of nano-encapsulated drugs, in the case of therapeutic applications, or specific dyes, in the case of colorectal cancer monitorization.

CEA-targeting technologies that were already produced, are mainly focus on monitorization of colorectal cancer evolution, and only a few addresses the specific guiding of drug delivery systems. The affinity of the ligands used for the functionalization of nanoparticle-based systems could also modulate the tendency of binding to the membrane-linked CEA or to the serum-available soluble CEA, secreted by tumour cells. Independently of the affinity of the ligand, the internalization into a cancer cell occurs by non-specific mechanisms, and the usage of less affinity ligands for CEA recognition is maybe the best choice when an intravenous administration is desired. Here, we envisage that the use of antibody fragments-decorated nanoparticles, with high affinity characteristics and likely to perform an oriented functionalization, might be a successful approach for CRC treatment and monitorization. Between the nanocarriers most suitable for this purpose, PLGA nanoparticles have a huge impact, due to its biocompatible and low toxicity features and is expected to sustain important advances in the near future.

Overall, we have highlighted the great potential of development CEA-targeting nanocarriers for drug deliver into colorectal tumours, which remains a poorly explored field of development.

Conflict of Interest

The authors declare no conflict of interest.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: ARS, MJO, BS

Participated in searching the literature: ARS

Prepared the images and tables: ARS

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Footnotes

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Legends for Figures

Figure 1

The carcinoembryonic antigen cell adhesion molecules (CEACAM) family.

Each molecule harbours one variable (IgV-like) N-terminal domain (rose ball), homologous to the Ig variable domain. The terminal N-domain is generally linked to constant domains (IgC2-like), that are represented here as the blue balls with the letters A and B. CEACAMs 5-8 are covalently bound to the membrane by a GPI linkage (blue arrows), whereas CEACAMs 1,3,4 and 19-21 use transmembrane domains. CEACAM16 is the only fully secreted protein. CEACAMs are generally highly N-glycosylated (green shapes).

Figure 2

Structure of conventional whole immunoglobulin and antibody fragments.

A) Conventional IgG has one Fragment crystallizable region (Fc) and two fragment antigen binding (Fab) regions, each one containing one Fragment variable (Fv) region. The two heavy (H, on blue) and Light (L, on green) chains contain the amino-ended Variable region (VH or VL, respectively), and the carboxyl-ended Constant region (CH1, CH2, CH3 or CL, respectively). The sites for antigen binding are given by three Complementary-Determining Regions, CDRs (the green arches on the amine-ending). The Fc portion is also glycosylated (yellow hexagons). The disulphide bridges (S-S) stabilize the 'Y' format of the Ig. B) Native antibody fragments. F(ab)'2, Fab', Fab and half-antibodies - hAb (~67kDa). C) Genetically-engineered antibody fragments. Single-chain variable fragments - scFv (~27 kDa), single-domain antibody fragments - sdAb (~13 kDa) and SS-Fc bispecific fragments (~80 kDa).

Figure 3

Most common reaction chemistries to conjugate antibodies to other structures.

A) The linkage between a carboxylated structure and the primary amines of scFv (antibody fragment) could occur by adding two crosslinkers: EDC and NHS, (or its more water-soluble form, sulfo-NHS). Generally, when applying EDC (step 1) is also added sulfo-NHS (step 2) to increase the efficiency of the reaction. There are also circumstances where the carboxylated structure is already activated by sulfo-NHS, forming a sulfo-NHS ester structure, and in this situation (starting on step 2) there is no need to add any crosslinker. B) In the linkage between a maleimide-ended structure and a thiolated scFv, maleimide works as the crosslinker and the X groups on it could be, most commonly, a simple hydrogen or preferably, any good-leaving group as a halogen. The thiol (-SH) and disulphide(S-S) groups on scFv should be previously reduced to guarantee that they are ready for conjugation.

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Tables

Table 1. Nanoparticle-based targeting systems to promising cell surface molecules for gastrointestinal cancer treatment and monitorization.

Receptor	Cell lines	Ligand	Formulation	Drug delivered	Remarks	Ref.
CEA	LS174T HCT-116	Monoclonal antibody (mAb)	Magnetic NPs	-----	Maghemite NPs conjugated to anti-CEA (~550 nm) had greater uptake by CEA^+ CRC cells. The biocompatibility of the system was confirmed.	(da Paz et al., 2012)
CEA and TAG-72	LS174T HT29	Anti-TAG-72 mAb and Anti-CEA mAb	Human serum albumin NPs		*In vivo* studies performed with LS174T and HT29 xenografts. NPs with ~120 nm had specific binding for mice CRC tissues.	(Margel, 2012)
EGFR	HCT-116 SW-480 HT-29 SW-620	Cetuximab-Fab' fragment	Liposomes	Oxaliplatin	Liposomes had ~120 nm, efficiency of encapsulation of ~32% and a loading capacity of ~65 µg/mg. Fab'-Liposomes induced cell-specific uptake, and cytotoxicity to $EGFR^+$ CRC cells.	(Zalba et al., 2015)
VEGFR	CT26	Polyclonal antibody	Dextran-coated iron oxide NPs	----	*In vivo* studies performed with CT26 xenograft. Anti-VEGF-NPs had 65 nm, demonstrated *in vivo* tumour targeting and efficient accumulation in tumour tissues.	(Hsieh et al., 2012)
CD44	Colon-26	Hyaluronic acid (HA)	PLGA NPs	Camptothecin (CPT) / Curcumin (CUR)	HA-functionalized PLGA NPs with ~300 nm, co-delivered Camptothecin (CPT) and Curcumin (CUR) (1:1) for CRC-targeted combination chemotherapy evidenced enhanced toxicity.	(Xiao et al., 2015)
CD44v6	MKN74 (gastric cancer cell line)	Fab (fragment antigen binding)	PLGA-PEG NPs	---	NPs of ~300 nm and tagged with the Fab had specific cellular binding. NPs coated with Fab($CD44v6^+$) showed negligible binding to negative cells, as the Fab($CD44v6^-$)-decorated NPs on the positive cells.	(Kennedy et al., 2018)
CD44v6	PANC-1 (pancreatic cancer cells)	Single-chain variable fragment (scFv)	Amphiphilic deblock copolymer of poly (ethylene glycol) and poly (D, L-lactide) [mal-PEG-PDLLA]	Arsenic trioxide (As_2O_3)	*In vivo* studies performed with PANC-1 xenografts. mal-PEG-PDLLA vesicles had ~200 nm and encapsulation efficiency of 65.8%. scFv-loaded-NPs (drug concentration of 8 mM), induced more apoptosis than the free drug or non-functionalized-loaded NPs.	(Qian et al., 2013)
Folate Receptor	HT-29	Folic Acid (FA)	PLGA NPs	5-FU	Nanoparticles had ~200 nm, encapsulation efficiency of ~30% and drug loading of ~6%. FA conjugation of ~47% was obtained using 1, 3-diaminopropane as linker. 5-FU loaded FA-PLGA NPs showed cell toxicity at 50µg/mL.	(Wang et al., 2015)
CA 19-9	Pancreatic cell lines: AsPC-1, BxPC-3-Luc, KP4, PK-59	L-fucose	Liposomes	Cisplatin	*In vivo* studies performed with AsPC-1 and BxPC-3-Luc xenografts. L-fucose-Liposome cisplatin-loaded had ~200 nm. The greatest cytotoxicity was observed when using 50µg/mL Fuc-Liposomes. Being these ultimate ones more cytotoxic than the free drug.	(Yoshida et al., 2012)
TfR	Caco-2	mAb	Liposomes	-----	Anti-Trf-NPs with ~100 nm, had 4.5-fold greater binding than the ones non-functionalized or coated with negative mAb.	(Harel et al., 2011)
DR-5	HCT-116	mAb	PLGA-PEG NPs	Camptothecin	*In vivo* studies performed with HCT-116 xenografts. Nanoparticles had ~200 nm, and association efficiency of ~18%. In mice treated with DR5-NPs, the malignant mass reduced ~35% over both PBS and control-IgG conjugated NPs.	(Schmid et al., 2014)

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Table 2. CEA-targeting systems for colorectal cancer monitorization and treatment in clinical trials.

Ligand	Conjugate	Interventions	Clinical indication	Route	Phase	State	Code	Sponsors
TF2 bispecific anti-CEA mAb	----	Pretargeted Radioimmunotherapy	Metastatic colorectal cancer	IV	I	Terminated	NCT01273402	Immunomedics, Inc.
Anti-CEA diabody	^{123}Iodine-Labeled cT84.66 Diabody	Immunoscintigraphy	Colorectal cancer	IV	I	Completed	NCT00647153	City of Hope Medical Center
SGM-101	NIR fluorochrome-labeled anti-CEA mAb	Surgical resection histopathology	Colorectal and pancreatic cancer	IV	I/II	Recruiting	NCT02973672	Surgimab
SGM-101	NIR fluorochrome-labeled anti-CEA mAb	Surgical resection histopathology	Colorectal cancer and metastases of patients undergoing surgery	IV	III	Not yet recruiting	NCT03659448	Surgimab
M5A	yttrium^{90} (90Y) DOTA anti-CEA monoclonal antibody M5A	Treatment: Irinotecan hydrochloride Leucovorin calcium Fluorouracil Bevacizumab Yttrium^{90} DOTA anti-CEA monoclonal antibody M5A	Metastatic colorectal cancer	IV	I	Completed	NCT01205022	City of Hope Medical Center
M5A	Cu^{64} (copper-64) anti-CEA monoclonal antibody M5A	Positron emission tomography (PET)	CEA-expressing cancers as the ones from gastrointestinal tract	IV	n.a.	Recruiting	NCT02293954	City of Hope Medical Center

n.a.: not applicable;
www.clinicaltrials.gov

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Table 3. CEA-targeted nanosystems for monitorization and therapeutic applications.

Ligand	Formulation	Linker	Drug	Features	Ref.
CEA aptamer	Combination of Silver nanoclusters (AgNCs) and gold nanoparticles (AuNPs)	Half-complementary DNA + CEA aptamer + half complementary DNA	_ _ _ _ _ _	Detects CEA within a range of 0.01-1 ng/mL. The CEA detection limit was 3 pg/mL. DNA-Au NPs had 15.4 ± 0.7 nm and -37.3 ± 1.5 mV. This method was validated by testing CEA in healthy human blood samples.	(Yang et al., 2015)
Amine modified CEA aptamer	Upconverting nanoparticles (UCPs)	CEA amine modified aptamer + hexanedioic acid (HAD)	_ _ _ _ _ _	The CEA detection occurred within a range of 4-100 pg/mL. The CEA detection limit was 1.7 pg/mL. The HSA-UCPs had 10-20 nm. The CEA aptamer was conjugated through carbodiimide chemistry.	(Li et al., 2016a)
mAb anti-CEA	Silica nanoparticles	SMCC $SM[PEG]_4$ EDC/sulfo-NHS PAMAM dendrimers	_ _ _ _ _ _	*In vivo* studies performed with LS174T xenografts. PAMAM dendrimer-conjugated particles had 71 nm. The CRC cell lines used for *in vitro* studies were LS174T, LoVo and HCT116. CEA-targeted PAMAM dendrimer-conjugated NPs had the highest binding to CEA comparing with the negative control.	(James P Tiernan, 2015)
mAb anti-CEA	Carbon nanotubes	BSA-fluorescein	Doxorubicin	A single SWCN had ~1 nm, AE of 87.5% (indirect method) and theoretical DL of 11.6 %. The weight ratio of Doxorubicin to oxidised SWCNs is 20:1. The carbodiimide chemistry was applied. CRC cell lines for in vitro studies: WiDr.	(Heister et al., 2009)
mAb anti-CEA	PLGA nanoparticles	PEG-COOH	Paclitaxel	NPs had ~ 200 nm and -10.4 mV with a low PdI. They had also a practical DL of 16.6% and AE of 99.4 %. Carbodiimide chemistry was applied and the NPs showed a sustained release up to 48h and had no cytotoxicity in the CRC cells. CCR cell line CEA^+ was Caco-2 and CEA^- was SW480.	(Pereira et al., 2018)
Sm3E (scFv)	Superparamagnetic iron oxide nanoparticles (SPIONs)	Dextran-OH Dextran-PEG-COOH	_ _ _ _ _ _	Sm3E was engineered with a C-terminal (6x His) tag and produced in yeast. The scFv K_D was 30 pM. Carbodiimide conjugation strategy was applied. CRC cell line CEA^+ was LS174T and Melanoma cell line CEA^- was A375M.	(Schmidt et al., 2008; Vigor et al., 2010)
shMFE (scFv)	PEG chain (5 kDa) Fluorescein Biotin Nitroxide spin label	Dibromomaleimide Dithiophenolmaleimide	_ _ _ _ _ _	shMFE has tropism to the same CEA epitope as Sm3E does and was also produced in yeast. The K_D of shMFE to CEA was 8.5 nM and the K_D of spin-labeled scFv in PBS was 1.91 ± 0.78 µM, while in plasma was 4.35 ± 1.27 µM and in whole blood was 6.46 ± 1.7 µM. The CEA detection limit was 100nM (spin labelled-scFv). Maleimide chemistry was applied. The PC cell line CEA^+ was CAPAN-1 and the melanoma cell line CEA^- was A375.	(Schmidt et al., 2008; Schumacher et al., 2013)
MFE-23 (scFv)	Carbon nanotube	1-pyrene-NHS ester Hexahistidine tag	_ _ _ _ _ _	Ni-NPs had 20-60 nm and are linked to nanotubes through an electrochemical technique. The scFvs have an hexahistidine tag in its C-terminal. The fragment was produced in bacteria.	(Young-Seop Lo, 2009)
SS-Fc	Anti-Flag-FITC	Histag Flagtag	_ _ _ _ _ _	*In vivo* studies performed with LS174T xenografts. SS-Fc was produced in bacteria. The Histag (6x His) and Flagtag (polypeptide chain) motifs were added to the C-terminal of anti-CEA-Fc and anti-CD16-Fc domain. The K_D was 0.195 nM (for CEA) and of 5.75 nM (for CD16). The SS-Fc had potent toxicity against CEA^+ cells HT29 and LS174T. The ovarian cancer cell line CEA^- was SKOV3.	(Li et al., 2016b)
hAb anti-CEA	Lipid-polymer hybrid NPs	PEG-Maleimide	Paclitaxel	hAb-NPs had 95 nm and -55 mV. The hAb-NPs had an IC50 of 251 nM and non-functionalized particles had an IC50 of 526 nM. The theoretical DL was 3.8 %. The maleimide chemistry was applied and NPs functionalized with hAb had more than 2-fold increase in toxicity comparing to naked NPs. The PC cell line CEA^+ was BxPC-3 and CEA^- was XPA-3.	(Che-Ming Jack Hu, 2010)
sdAb-CEA	Quantum Dots (QDs)	Sulfo-SMCC PMPI	_ _ _ _ _ _	The K_D was 8.3 nM and sdAb-QDs had 11.9 ± 2.9 nm. sdAb was enginnered with a 6-Histidine tag chain in its C-terminal (sdAb-C17 his6Cys). Produced in bacteria. The CRC cell line CEA^+ was MC38CEA and $CEA^-$was MC38.	(Sukhanova et al., 2012; Ramos-Gomes et al., 2018)

AE, Association Efficiency; CRC, colorectal cancer; DL, Drug Loading; His, Histidine; K_D, Equilibrium Dissociation Constant; NPs, nanoparticles; PC, pancreatic cancer; PdI, polydispersity index; scFv, single-chain variable fragment; SWCN, single-walled carbon nanotube;

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Figures

Figure 1.

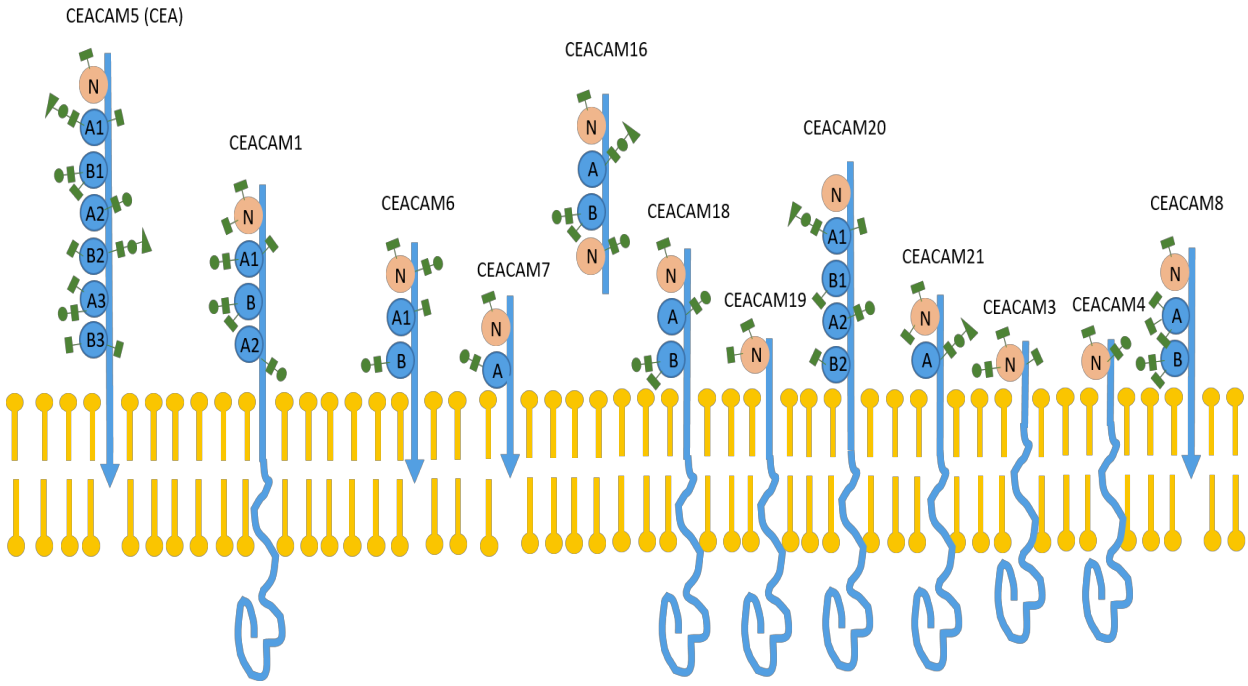

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Figure 2.

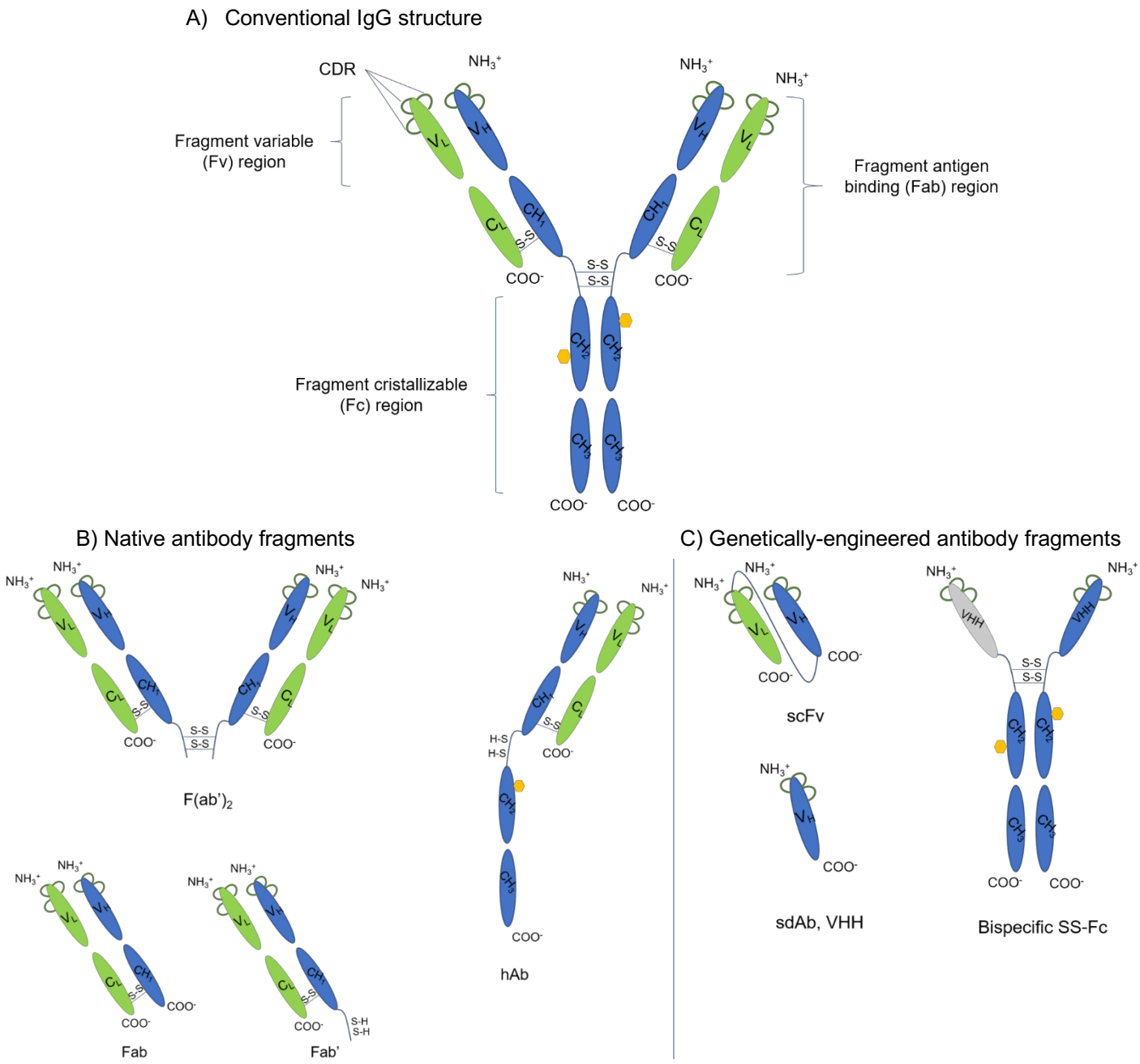

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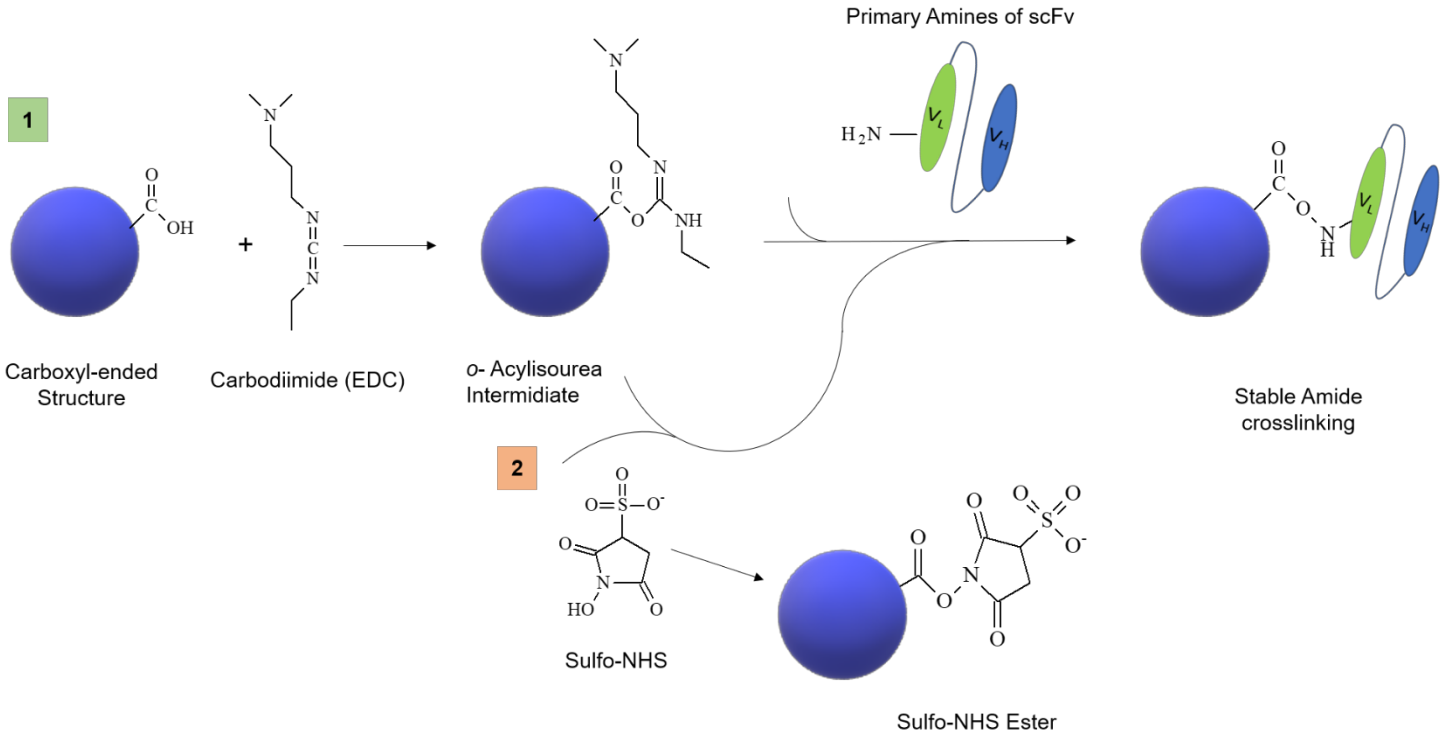
Figure 3.

A) Crosslinking carboxyl-to-amine functional groups by using EDC (1) and sulfo-NHS ester (2) reaction scheme



B) Crosslinking maleimide-to-sulfhydryl functional groups by using maleimide reaction scheme