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Construction and testing for anticancer effect of a TheraCour Biopolymer

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Abstract

Polymeric micelles present the most presentable as it can be tailored according to the needs of specific targeting and make it functional. The TheraCour polymers are designed as linear, uniform, homopolymers Comprising Polyethylene Glycol (PEG) as a monomer unit that is hetero-chemically functionalized and covalently connected with aliphatic chains as ligands. For cancer applications, TheraCour polymers bearing folic acid or EGF-peptide as ligands targeting Folate-receptor-hyper expressing, or EGFR-enriched cancers, respectively, have been synthesized. Encapsulation of many hydrophobic drugs such as camptothecin, paclitaxel, colchicine and others has been performed. *In vitro* anticancer effects are shown with these nanopolymers on two lung cancer cell lines and three breast cancer cell lines.

Introduction

An engineered nanoscale materials is an innovative prototypes to be used for bio-medical applications and optimized therapy. Due to their unique features, including a large surface area, structural properties, and a long circulation time in blood compared with small molecules, a plethora of nanomaterials has been developed, with the potential to revolutionize the diagnosis and treatment of several diseases, in particular, by improving the sensitivity and recognition ability of imaging contrast agents and/or by selectively directing bioactive agents to biological targets.

Typically, cancer therapies involve the systemic or oral administration of drugs into the body, both of which routes can damage healthy tissues by significant off-target accumulation and thereby generate serious side effects. Further, the off-target accumulation limits the dosage that can be administered. To overcome these limitations, various targeting strategies are being investigated.

An extensively investigated polymer is Poly (Lactic-Co-Glycolic Acid) (PLGA), synthetic thermoplastic aliphatic biocompatible polyester. There are specific formulations based on PLGA and its related homopolymers; Poly (Lactic Acid) (PLA) and poly

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(glycolic acid) (PGA), which are considered safe and a few biodegradable polymer products have been approved by the US Food and Drug Administration (FDA) as well as by the European Medicines Agency (EMA) for pharmaceutical application [1]. Generally, PLA, PGA, or PLGA polymers in different forms and shapes are used for creating "nanocapsules" of chemotherapeutics (i.e. essentially solid-form drug depots at nano-scale) which is akin to using hand-grenades (nanocapsules) in place of bullets (the small chemical drug as is). While they provide protection from stomach acid, enabling drug delivery further downstream in the gastrointestinal tract, they generally lack the specificity of receptor targeting, and if not properly formulated, can even increase the risk of localized side effects [2].

Addressed targeting

Targeting of specific over expressed receptors by an enveloping nanostructure for the purpose of delivering a cargo of chemotherapeutics into cancer cells can reduce the risk of off-target effects. Nanostructures most used for accomplishing this effect have been liposomes, lipid nanoparticles, and their pegylated versions, dendrimers, and others, although certain classes of polymeric micelles may offer unique advantages as discussed further below. This is referred to as address-based targeting to distinguish it from specific enzyme inhibitors that have been generally described as targeted therapy in the cancer field (e.g. Kinase inhibitors, PARP inhibitors, DNA methylation and histone acetylation modifiers, etc.).

Addressed Targeting can be accomplished using antibodies, natural ligands, natural-ligand-mimetics, specific peptides, Monoclonal Antibodies, PEGylation (covalent attachment of PEG chains, typically methoxy-PEG, mPEG) as well as small chemical ligands often derived from structure-based drug design.

Clinical development of nanovehicles for address-targeted therapies

To date, only three targeted liposomes and four targeted polymeric nanoparticles have progressed in clinical development. The prototype that reach clinical trials was MCC-465, a new generation of liposome-encapsulated doxorubicin with a surface covered by both PEG and antigen-binding fragments [F(ab')2] to confer immune shielding and targeting, respectively, that is used in the treatment of human stomach cancer [3].

Another site-directed liposome is SGT53-01, a TfR-functionalized nanoformulation containing a chain antibody fragment (TfRscFv) as the targeting ligand. It has been designed to carry the p53 tumor suppressor gene to cancer cells, and is currently undergoing Phase I clinical trials in combination with doxorubicin for the treatment of solid tumors [4,5].

Despite the progress made in the clinical development of liposome-based nanocarriers, there are several issues that limit their wider consideration as a main drug delivery platform, including the difficulty in modulating their drug release in vivo, the limited amount of drug that can be loaded, possible oxidation of liposomal phospholipids, and the inherent instability of liposomes. In contrast, polymeric nanocarriers demonstrate superior stability in vivo as compared to liposomes, provide a high drug-loading capacity, and enable both controlled and triggered release of drugs. Therefore, polymer-based nanomaterials have the potential to provide solutions for a range of problems in nanomedicine [6-10].

In the field of breast and prostate cancer the application of liposomes has been increasingly common [11-13]. Multiple paclitaxel liposomes have been demonstrated to have higher antitumor efficiency and improved bioavailability compared to free paclitaxel [14].

Liposomal doxorubicin has been proven to reduce cardio toxicity and has comparable efficacy in breast cancer [15,16]. Doxil, a pegylated form of liposomal doxorubicin was the first such drug approved for cancer treatment, leading to an explosion of research and development in this field. Furthermore, liposome-based nanosystems have also offered an option for delivering drug combinations, which can enhance the therapeutic effect [17,18], and even reverse the drug resistance [19]. Nowadays, many liposome-based drugs have entered into clinical use for cancer treatment [20].

Polymer-based NPs are another type of NP with specific structural arrangements for drug delivery formed by different monomers [21]. All polymeric nanopharmaceuticals benefit from the "Enhanced Permeability and Retention (EPR)" effect. Briefly, as tumors grow, new blood vessels are formed to provide oxygen and nutrients to the growing tumor (neo-angiogenesis) that are more porous than normal blood vessels and allow cross-passage by large polymeric nanopharmaceuticals from blood into the tumor space. The polymeric nanoparticles can be designed to be small enough to enter the cancer cell, but are too large to then be shuttled out by the tumor cell's transport pumps. They can then disintegrate over time while dynamically releasing the active drug payload within the tumor cell.

Research on nanotechnology and paclitaxel (PTX) nanoformulations have enhanced the medical outcome of chemotherapy and cancer [22]. Paclitaxel is a highly effective anti-tubulin chemotherapeutic agent, which is widely employed for the treatment of numerous malignancies. Nanomedicine forms of PTX have been developed both by compounding PTX into polymers and by conjugation to polymers. The objective is to improve bioavailability and reduce the incidence of adverse effects. Many natural and synthetic polymers have been used for PTX nanoencapsulation, including PLGA NPs, PLA NPs, chitosan NPs and polymeric micelles [22]. The in vivo tumor inhibitory activity of paclitaxel-loaded PLGA nanoparticles was markedly better in transplantable liver tumors [23]. In the live small animals, laser confocal scanning microscopy and fluorescence imaging showed the location of the fluorescence-labeled paclitaxel-A10-3.2-PLGA and nanobubbles.

Opaxio (Paclitaxel polyglumex, Xyotax, CT-2103, Cell Therapeutics) is a covalent conjugate of PTX to poly-L-glutamic acid, that has gone through multiple clinical trials for the treatment of a variety of cancers including prostate, breast, ovarian, colorectal, and lung cancers [24]. The drug was shown to concentrate at tumor sites due to the EPR Effect. In clinical trials it failed to meet the primary endpoint of improved survival, although side effects were significantly reduced compared to the standard control arm therapy models. Its development appears to have been stopped.

Focusing on one of the first clinically tested targeted nanomedicines, a major effort went into the development of BIND-014 [25], a new docetaxel formulation developed by a team led by Langer and Farokhzad at the Massachusetts Institute of Technology, Harvard Medical School, and BIND Therapeutics, as a programmable nanomedicine that entered Phase II clinical testing for the treatment of patients with solid tumors (approved

for breast cancer, head and neck cancer, and gastric cancer).

With regard to targeted polymeric nanoformulations, CALAA-01 was the first nanotherapeutic to reach clinical development for siRNA delivery in 2008 [26]. This nanosystem consists of TfR-targeted cyclodextrin-based PEGylated nanoparticles containing siRNA, and is capable of reducing expression of the M2 subunit of ribonucleotide reductase. The safety of CALAA-01 was evaluated in a Phase I clinical trial by intravenous administration to adults with solid tumors refractory to standard of care therapies [27].

Several systems to provide address-targeted therapy of this type have been designed and have undergone testing. Micelles and liposomes offer another option for delivery of chemotherapeutic agents. Micelles, in particular, provide an important tool to make aqueous-insoluble drugs soluble due to their hydrophobic core and hydrophilic shell. If the micelle's surface is further PEGylated, it increases the ability of the nanocarriers to get through fenestrated vasculature of tumors and inflamed tissue through passive transport, thus resulting in higher drug concentration in tumors. As of now, several polymeric micelles containing anticancer drugs, NK012, NK105, NK911, NC-6004, and SP1049C are in clinical trials [28], and one such system, Genexol-PM (paclitaxel) is approved for breast cancer patients [29].

TheraCour polymeric micelle approach for anticancer regimen

TheraCour platform polymer is a self-assembling, uniform, tailorable linear homopolymer that comprises Polyethylene Glycol (PEG) within its monomer unit which is heterochemically functionalized with a specially designed linker unit so that covalently connected aliphatic chains are suspended from it and separately site-targeting ligands are also covalently attached to it [30-32] (Figure 1). This simple scheme results in a polymer that is like a half-biological membrane. In aqueous systems, it self-assembles into micelles with hydrophobic, flexible core region made of the lipid chains, hydrophilic ligands directing outwards into the aqueous milieu ready to seek their partners, connected together by the corona of PEG.

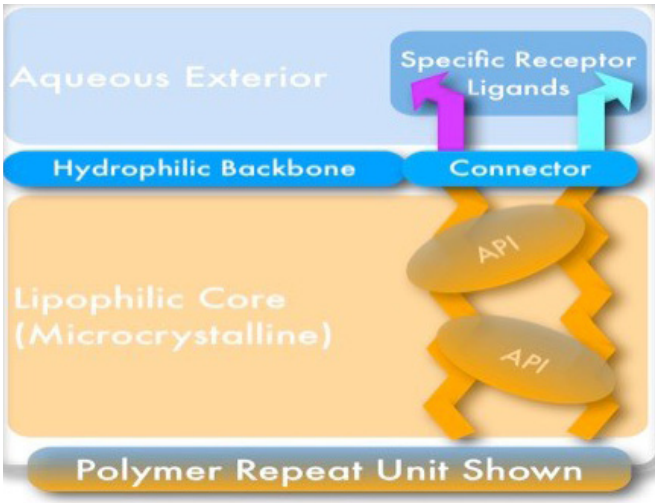

Figure 1: Graphic Design of TheraCour Polymeric Micelle. A schematic representation of a TheraCour Bio-polymer designed in such a way that an API (Active Pharmaceutical Ingredient) present in lyophilic core with repeat-unit is connected to a specific receptor ligands present in aqueous exterior area.

Upon binding of the micelle to a cellular receptor sought out by the ligand, multiple interactions with cellular receptors can take place resulting in increased avidity, due to the regular presentation of ligand at each monomer unit. This forces the corona close to the cell membrane and may initiate lipid-mixing of the flexible pendant interior lipid chains of the micelle with the flexible lipids of the cell membrane, leading to passive fusion. Alternatively, receptor-mediated endocytosis can take place at properly chosen receptors. These processes would result in site-specified or address-targeted delivery of the encapsulated drug payload content of the micelle. As encapsulated rather than covalently immobilized, the drug payload can immediately go to work and does not have the latency of the need to be released from the polymer backbone in a covalent system. This polymer can effectively encapsulate many types of chemotherapeutic APIs, target the cancer cell based on the selected ligand, and thereby result in effective anticancer activity. The graphical model of anticancer mechansim of TherCour platform technogy is shown in Figure 2.

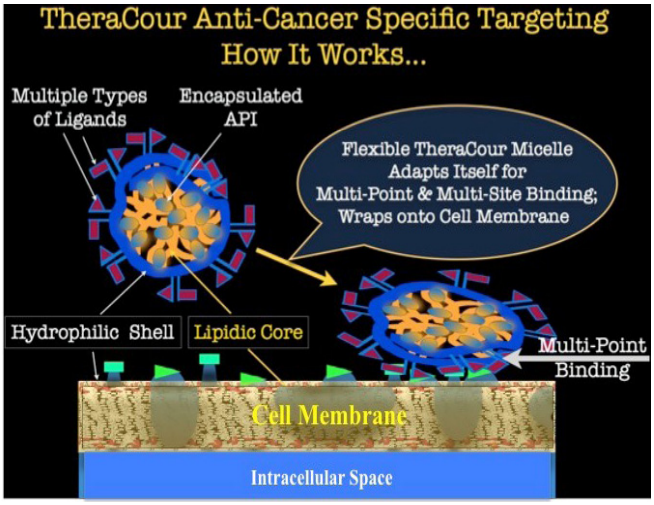

Figure 2: A Graphical Representation of TheraCour Anti-Cancer Mechanisms of Specific Targeting. Flexible TheraCour micelle adapts itself for multi-point and multi-site binding, and then wraps onto cell membrane.

Materials and methods

Culture of human cancer cell lines

Two human lung cancer cell lines, A549 and H44, and two human breast cancer cell lines, SKBR3 and BT474 were procured from ATCC (Manassas, VA). The growth and maintenance of those cells were conducted with their appropriate growth medium plus 10% FBS (Gibco). at 37°C in a 5% CO_2 humidified incubation chamber. Sub-culturing and passaging of the cells were done by Accutase, a cell dissociation reagent, (ATCC, Manassas, VA), into single cells.

Treatment of cells with our test components

Cells ($1x10^3$/ well) were plated in 96- well plates. Next day, treatment was done with an untargeted TheraCour polymer (i.e. no receptor ligand), a targeted TheraCour polymer (in this case, folate), and with or without encapsulated API (camptothecin, CPT).

Proliferation assay by cell-glo luminiscence reagent

Proliferation of cells was detected using the Cell-Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI). Next day '0 day' reading from a sample well (in triplicate) was taken and the rest of the wells are treated with our actives.

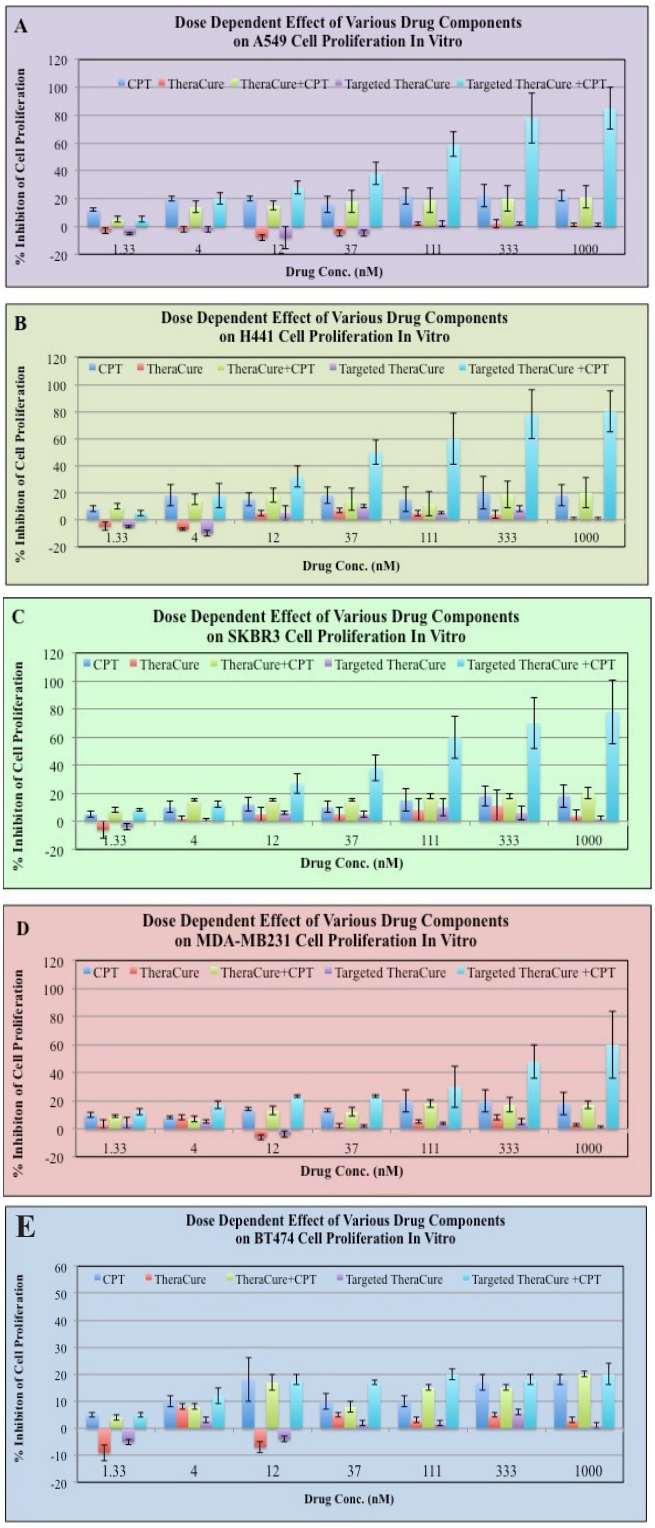

Figure 3: Cell Proliferation of Cancer Cell Lines is Most Inhibited by a Folate-Targeted TheraCour Polymer Delivering Camptothecin (CPT).

Panel A: Dose dependent effect of various drug components on lung cancer cell Line A549 *in vitro.*

Panel B: Dose dependent effect of various drug components on lung cancer cell Line H441 *in vitro.*

Panel C: Dose dependent effect of various drug components on breast cancer cell Line SKBR3 *in vitro.*

Panel D: Dose dependent effect of various drug components on multi-drug resistant breast cancer cell Line MDA-MB231 *in vitro.*

Panel E: Dose dependent effect of various drug components on triple negative and multi-drug resistant breast cancer cell Line BT-474 *in vitro.*

Every alternate day cell growth was measured using the Cell-Titer-Glo- kit until for 6 days. Luminescence was read using the BioTek plate reader [33]. Results were expressed as percentage of vehicle-control cells. Results presented are Mean ± SD from three separate experiments done in triplicate.

Cell counting

Cell count and viability measurements were done by staining the cells with Trypan Blue and then counting the number of viable cells with the Countess TM automated cell counter (Invitrogen).

Statistical analysis

The t-test and one-way analysis of variance were used for statistically analyzing the data. All values are expressed as the mean ± SD. P values <0.05 were considered significant. Results are representative of at least three experiments.

Results

Figure 3 shows the cell proliferation data of two lung cancer cell lines, A549 (panel, A) and H441 (panel, B), and two breast cancer cell lines, SKBR3 (panel, C), MDA-MB231 (panel, D) and a multi-drug resistant line BT474 (panel, E) in presence of an untargeted TheraCour polymer (i.e. no receptor ligand), a targeted TheraCour polymer (in this case, folate), with or without encapsulated API (camptothecin, CPT).

The enhancement in effectiveness of folate-targeted TheraCour-encapsulated delivery of Camptothecin over that of CPT by itself in these cell culture studies is significant, enabling 75-90% inhibition at non-toxic concentrations of the drug, wherein CPT alone reached less than 20% inhibition, in four of the cancer cell lines; such high levels of inhibition are hardly ever reached with no cytotoxicity. The exception was that the folate-TheraCour-CPT drug showed 20% inhibition in Multi-drug-resistant breast cancer cell line BT474, which was still 10X greater than that from CPT alone (panel, E). These highly promising results need to be taken further into animal model studies and, if confirmed, then into full-fledged drug development.

Discussion

Traditional cancer chemotherapy is cytotoxic to cells, meaning it can damages healthy cells in addition to cancer cells, whereas targeted therapy affects only cancer cells but not the normal, healthy cells. In brief, looking for targeted therapy using nanotechnology becomes evident in cancer therapeutics. Recent advances in nanotechnology and biotechnology have contributed to the event of engineered nanoscale materials as innovative prototypes to be used for biomedical applications and optimized therapy. However, several obstacles, including difficulty in achieving the optimal combination of physicochemical parameters for tumor targeting, evading particle clearance mechanisms, and controlling drug release, prevent the interpretation of nanomedicines into therapy. Despite of these advantages, recent efforts are focused on developing functionalized nanoparticles for delivery of therapeutic agents to specific molecular targets overexpressed on different cancer cells. Particularly, the mixture of targeted and controlled-release polymer nanotechnologies has resulted in a very new programmable nanotherapeutic formulation of docetaxel, which recently entered in clinical testing for patients with solid tumors.

Nanotechnologies are having a major impact on drug delivery, and over recent years, several first-generation therapeu-

tic nanoproducts are moving ahead towards clinical development. Particularly, targeted polymeric nanoparticles, capable of increased cell uptake and enhanced accumulation in target tissue, are often successfully obtained by attaching specific binding entities onto the surface of the nanoparticles. Therefore, active targeting, together with other targeting-based approaches, is envisaged to produce an efficient strategy, and a number of other ligand-targeted nanotherapeutics are either approved or under clinical evaluation, resulting in second-generation nanomedicines.

A polymeric drug delivery nanovehicle containing the chemotherapeutic agent docetaxel, which is approved to be used within the treatment of several common cancers, including breast, lung, and prostate. To date, other complex targeted nano-systems addressed to varied cancers, also combining diagnostic and therapeutic agents, or that may trigger drug release at the target site when exposed to external stimuli, are currently in clinical development.

The TheraCour platform provides an anticancer effect as shown in this manuscript, and further allows an extremely wide range of tailorability according to the nature of cancer:

a) Different ligands can be chosen to attack correspondingly different targets.

b) The hydrophobic/hydrophilic balance can be beneficially and systematically altered by choosing appropriate lipid length, and balancing PEG-monomer chain length. Increasing the lipid chain length can result in a more hydrophobic polymer that would form more stable and larger micelles, and would be more suitable for dermal topical delivery as a cream or as an ointment formulation. In contrast, very short lipid chains would make water-soluble yet micelle-forming polymer wherein the tageting ligand itself behaves as a polymer-conjugated drug, and the short lipid chains merely assist in conformational stability and adherence to cell membrane once attachment takes place, albeit with diminished encapsulated API cargo capacity. A medium between these illustrations would be well suited for drug delivery as an injectable drug. Thus the final usage (route of administration informs the design itself.

c) The connector can be modified to tailor the rate of release of the API by controlling the rate of depolymerization advantageously using natural enzymes such as esterases in the bloodstream, proteases, caspases, and others in the tumor vicinity.

d) The connector can be modified to present a suitable net electrical charge state. A net negative electro osmotic potential on the micelles is beneficial for keeping the drug solution stable and keeping it from agglomerating. A strong negative zeta potential would result in reduction in non-specific binding to cells thus minimizing off-target effects. Adding spatially designed positive charges within the milieu of a net negative zeta potential can lead to interesting effects such as concentration into extracellular matrices and thus proximity to the tumor.

e) The polymerization can be controlled, within limits of the Flory equation, to provide desirable clearance characteristics. Thus, an overall molecular weight (including encapsulated API contribution) greater than about 10 KDa is generally expected to minimize renal clearance until after polymer degradation, thereby limiting renal injury, a key issue in several chemotherapeutics. At very large molecular weights, the drug would only circulate or deposit in the blood stream and only slow introduction into extra vascular space upon polymer chain degradation would be expected. The optimal molecular weight lies somewhere between these extremes, to minimize renal clearance and simultaneously enable EPR-based concentration in extra vascular tumor space.

f) More than one different APIs can be encapsulated, enabling combination drug therapy within single delivery, albeit in a fixed-dose ratio mode.

g) More than one different ligand targeted to different receptors can be attached. Choosing two different ligands targeted at the same receptor but at different locations can help select the stereo-pose of the receptor (e.g. activated versus native, or open versus bound to natural ligand) to achieve specificity. Choosing mutiples of the same ligand at each connector can lead to receptor multimerization, kicking off intracellular pathways. Choosing two different ligands directed at two different receptors can significantly enhance tumor selectivity, and further, can also enable hetero-dimer formation to kick-off intracellular pathways.

h) Limited cross linking can be introduced into the linear polymer chain if depot-type controlled release properties are desired.

i) Signal-based release of contents can be tailored in using appropriate connector chemistry, typically, pH-sensing, or esterase or protease-specific functions can be incorporated.

We have generally chosen small chemicals, small peptides (such as fragments modified from natural ligands), or small peptido-mimetics as the targeting ligands, as their sizes are in proportion with the polymer size constraints discussed already. Antibodies tend to be very large and are only suitable when high affinity antibodies are attached at one or both chain ends of the polymer.

Nonetheless, selection of appopriate cancer specifc ligands to be attached with our Biopolymer and encapsulaton of one or more anticancer drugs in its core vessels can make a magic bullete for cancer therapy.

Declarations

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Conflict of interest: All the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Certificate: We certify that all the Figs and Schematic designs that are presented in this manuscript are our own. No Animals are involved in these studies.

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