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The effect of linker type and recognition peptide conjugation chemistry on tissue affinity and cytotoxicity of charged polyacrylamide

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ABSTRACT

To increase colonoscopy competence in ambiguous situations (e.g. the existence of flat polyps), an explicit *in situ* (at real time) diagnosis at the molecular level is required. We have previously shown that the affinity of fluorescent cationic polyacrylamide (Flu-CPAA) to malignant regions in the colon mucosa can be improved by conjugating the recognition peptide EPPT1 to the polymer backbone (to form Flu-CPAA-Pep). Using another recognition peptide, namely VRPMPLQ, we elucidated in the present study the effect of linker type and conjugating methods on Flu-CPAA-VRPMPLQ cytotoxicity and on its affinity to cell lines as well as human colorectal cancer (CRC) biopsies.

In order to derive the relationship between the response variable and the experimental factors in a minimal set of experiments, a computerized statistical design of experiment (DoE) strategy was implemented. Data were collected in a six-factor factorial design to study the effect of experimental factors (independent variables) on the ability of the Flu-CPAA polymers to bind specifically to the colon cancer cell lines or the human biopsies (the response).

It was found that the presence of VRPMPLQ on the Flu-CPAA improved the polymer's affinity to the human CRC biopsies and to the colon cancer cell lines representing stage B in the Duke severity staging system. The cytotoxicity of Flu-CPAA with high charge density was reduced after conjugated with VRPMPLQ. The replacement of Ahx linker by PEG linker of similar length did not affect the affinity to the human biopsies, nor did it affect cytotoxicity. However, elongating the PEG linker reduced the *in vitro* affinity to the colon cancer cell lines and to human CRC biopsies. Changing the conjugation method from condensation (amide bond formation) to the click conjugation method did not affect the affinity properties of the polymers. It did reduce, however, the polymer cytotoxicity. We suggest that Flu-CPAA-Pep, with the VRPMPLQ peptide as a recognition moiety, could serve for early diagnosis and screening of CRC patients during endoscopic procedures.

1. Introduction

Early diagnosis and accurate screening of colorectal cancer (CRC), the third most common diagnosed cancer and the second leading cause for cancer in the USA [1], is crucial for the prevention as well as rational treatment of the disease. If diagnosed at early enough stages (i.e. while still localized in the colon epithelium), patients' five year survival rate can reach 90%. This value decreases to 68% when the disease transforms to a regional status and to 10% when distant metastases are identified [2]. Still, since only 5% of colorectal adenomas progress to malignant polyps, full ablation procedures and derived treatments may be associated with the cost of major over-treatment [1]. For example, in colonoscopy, serrated polyps are of major interest due to their likely potential for malignant transformation. However, their miss rates may be higher than adenomas because they are often flat, become flat with air insufflation and, thus, are more difficult to distinguish from the surrounding normal mucosa [3,4].

Abbreviation: Ahx, aminohexanoic acid; CPAA, cationic polyacrylamide; CRC, colorectal cancer; DoE, design of experiment; Flu-CPAA, CPAA tagged with Cy5; Flu-CPAA-Pep, Flu-CPAA conjugated to VRPMPLQ; Flu-CPAA-Pep-Scr, CPAA conjugated to the scrambled sequence VPQLRPM; SPPS, solid phase peptide synthesize.

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Apparently, screening procedures should make use of typical biomarkers, such as disease-dependent overexpressed proteins, to enhance differentiation resolution [5]. Alas, less than 10 biomarker molecules believed to be differentially expressed in human cancer are approved by the FDA for diagnostic purposes due to lack of adequate sensitivity and specificity [6]. Out of these biomarkers, serum carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), epidermal growth factor receptor (EGFR), and vascular endothelial growth factor (VEGF) are used as targets, predominantly to monitor the disease status after diagnosis due to inadequate specificity [5,7]. For this reason, intensive efforts are being invested to identify appropriate biomarkers that could serve as targets for particular diagnosis in the colon mucosa. The successful modality should include a targetable vehicle composed of a recognition moiety (an antibody or a peptide) and a fluorescent probe at a spectrum range with minimal background interference. This should be complemented by a suitable endoscopic device, such as high definition filter-aided colonoscopy or confocal laser endoscopy, representing the progress in advanced optics for medical imaging in recent years [8].

In terms of recognition capabilities, low molecular weight peptides are advantageous over monoclonal antibodies. The latter possess much higher molecular weight, shorter body elimination half-life, and immunogenicity. In general, the binding kinetics of recognition peptides is rapid (with reasonable tissue distribution patterns), they are easy to prepare, and their tagging with fluorescent dyes is straightforward [9]. Polymer-peptide conjugates are easy to engineer via the carboxyl or amino end groups of the peptides, enabling an assortment of suitable linkers and conjugation methodologies. Accordingly, there is much to explore on the relationship between linker types and linking chemistry and the polymer-peptide conjugates' recognition capabilities, trafficking properties, and cytotoxicity [10,11].

In the present study we suggest a polymeric imaging platform for early detection of colon dysplasia. In the past, we used the recognition peptide EPPT1 that targets the cell transmembrane under-glycosylated MUC-1 (uMUC-1) [12]. Here we employ the adenoma- specific heptapeptide sequence, valine-arginine-proline-methionine-proline-leucine-glutamine (VRPMPLQ), which was found to bind with high affinity and specificity to fresh human colonic adenomas, although not demonstrating full homology to ligands for known receptors [9].We prepared and conjugated this peptide to the cationic polyacrylamide (CPAA) which was shown in the past to bind to cells lines and tissues with affinities that correlated with the charge density of the CPAA and the metastatic stage of the cells and malignant tissues [13].

The specific goals of the study were: (*a*) Prepare CPAA and conjugate it to the Cy5 fluorescent dye to form Flu-CPAA, (*b*) Prepare VRPMPLQ and conjugate it to the Flu-CPAAs to form Flu-CPAA-Pep, (*c*) Test the cytotoxicity of the Flu-CPAA-Pep and its ability to bind specifically to colon cancer cell lines, (*d*) Test the effect of linker (used to conjugate VRPMPLQ to the Flu-CPAA backbone) type and length on the affinity properties of the vehicle to colon cancer cell lines and human biopsies, (*e*) Examine how the conjugation method (condensation, to form amide bond vs click reaction, to form a triazole bond) affects the affinity properties and cytotoxicity of Flu-CPAA-Pep, (*f*) Conduct a preliminary proteomics study in order to identify the biological target of VRPMPLQ and (*g*) Implement a computerized statistical design of experiment (DoE) strategy to derive the relationship between the response variable and the experimental factors.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma, St. Louis, MO, USA, unless otherwise stated. All solvents (Bio Lab, Jerusalem, Israel) were of analytical grade; Water was purified by reverse osmosis; Sodium carbonate anhydrous, *N,N* diisopropylethylamine (DIEA), piperidine, trifluoroacetic acid (TFA) and *N,N* dimethylformamide (DMF) were purchased from Bio Lab, Jerusalem, Israel; Acryl amide and triethylsilane were purchased from Fluka, Seelze, Switzerland; O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was purchased from Anaspec, Fremont, CA, USA; Benzoyl peroxide and molecular sieve (0.4 nm beads) were purchased from Merck, Darmstadt, Germany; 2.6-dimethylpyridine (Lutidine) was purchased from ABCR GmbH, KG, Karlsruhe, Germany; Polylactic acid (PLA, 100DL MW = 115 KDa) was purchased from Lakeshore Biomaterials Birmingham, AL, USA; Poly(vinyl alcohol) (PVA) (88% mole hydrolyzed, MW = 25 KDa) and linear polyethylene imine (Mw = 2.5 KDa and 250 KDa) were purchased from Polysciences Warrington, PA, USA. All amino acids were purchased from Gl Biochem, Shanghai, China. Cytokeratin 1st antibody (Ab), mouse monoclonal Ab pan-clones AE1&AE3 and the wash buffer were purchased from Zymoted Systems GmbH, Berlin, Germany; The 2nd Ab was F(ab)2 donkey anti mouse IgG conjugated to Dylight 488. It was purchased from Bethyl laboratories INC, Montgomery, Al, USA; DAPI Fluoromount-G was purchased from Southern Biotech, Al, USA; Deuterium oxide (D_2O), dimethyl sulfoxide (DMSO) D_6 chloroform D and methanol D_4 were purchased from Cambridge Isotope Laboratories, USA; Slide A lyzer™ GT dialysis cassettes, Dithiobis (succinimidyl propionate) (DSP), Carboxylink™, immobilization kit, Pierce IP lysis buffer and Halt protease and phosphatase inhibitor cocktails (ThermoFischer Scientific, Rockford, IL, USA) and Nucblue fixed cell stain ready reagent, DAPI special formulation was purchased from Thermo Fisher Scientific- Life Technologies, Eugene, OR, USA; 4-(dimethylamino) pyridine was purchased from Alfa Aesar, Ward Hill, MA USA; PEG linkers were purchased from Chem Pep, Welington, USA; Cy5 NHS ester was purchased from Lumiprobe, Florida, USA; Polyoxyethylene 20 sorbitan (Tween 20) and ehylenediaminetetraacetic acid (EDTA) disodium salt dehydrate were purchased from J.T. Baker PA, USA; Potassium chloride analytical and sodium chloride analytical were purchased from Frutarom, Haifa, Israel; Sodium carbonate anhydrous, granular was purchase from Mallinckrodt Chemicals, Phillipsburg, NJ; 1-Ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride, anhydrous (EDAC) was purchased from Chem-Impex International Wood Dale, IL, USA.

FTIR measurements were conducted with Nicolet™ iS™10 FT-IR Spectrometer (Thermo scientific Waltham, MA, USA). X-ray photoelectron spectroscopy (XPS) measurements were performed on a Kratos Axis Ultra X-ray photoelectron spectrometer with monochromatic Al Ka (1486 eV) X-ray source of the Unit for Nanoscopic Characterization, the Harvey Krueger Center for Nanoscience and Nanotechnology, the Hebrew University of Jerusalem. Proton NMR was recorded on a 300 MHz Brucker NMR using deuterated solvents as internal standards. Mass analysis was performed by LCMS (Finnigan LCQ duo, ThermoQuest Corporation, CA, USA), MALDI-TOF (Voyager De Pro, Applied Biosystems, CA, USA). Gel permeation chromatography was conducted in Multiangle laser light scattering (DAWN-F DSP Spectrophotometer, Wyatt, USA). Microscope images were taken by Cytation 3 imaging reader (Biotek, Winooski, USA), confocal microscope (LSM 710 processing T-PMT, Zeiss, Omaha, USA) and fluorescent microscope eclipse Ti-E (Nikon, Melville, NY, USA). Quantification of the microscope fluorescent images was performed by Image-Pro analyzer program (Version 7, Media Cybernetics, Rockville, MD, USA).

2.2. Cell lines and culturing

HT-29, SW-480 (both represent Stage B colon cancer severity in the Duke staging system), SW-620 (represent Stage D colon cancer severity in the Duke staging system, with increased expression of sialic acid) and IEC-6 (from non-transformed rat small intestine epithelium with characteristics of crypt mucosa; employed as control cells due to their low expression of sialic acid) cells [14] were purchased from American Type Culture Collection, Manassas, Virginia, USA. All cells were cultured (37 °C, 5% CO_2), in DMEM medium supplemented with 10% fetal calf