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14. ABSTRACT
The purpose of this project is to develop a new process for identifying breast cancer. This process should be at least as convenient as mammography for the patient, more sensitive for detection of even the smallest and earliest tumors, and more accurate in distinguishing tumors from normal tissue. First, the tumor site is marked by entrapment of a PEG-conjugate, due to the EPR effect. Based on the longer retention of the PEG-conjugate marker in tumor versus normal tissue, a second conjugate is administered. This second conjugate will chemoselectively interact with the first conjugate to form insoluble microgels only in tumors. Alternating cycles of the 2 conjugates should result in increasingly larger microgels. These microgels can then be used as targets to deliver another chemoselective reagent for detection (imaging) or for therapy. In this first year of the grant, we have developed procedures for synthesizing the various gel-forming conjugates needed for these studies. We have also established a syngeneic mouse model assay and have carried out pilot vivo experiments.

15. SUBJECT TERMS
chemoselective binding, fluorescence resonance energy transfer (FRET), enhanced permeationand retention (EPR), poly(ethylene glycol), PEG, 4T1 cells, doxorubicin, prodrug

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INTRODUCTION

The purpose of this project is to develop a method to detect breast cancer with greatest possible accuracy, smallest possible tumor size and least stress and inconvenience to the patient. We propose to combine several different techniques into a three-step process to accomplish this goal.

(1) The first goal is to place a "marker" on each tumor. For this purpose, we will use the "enhanced permeation and retention" (EPR) effect (Maeda et al., 2000), which might be universal for all tumors. Our markers will be optimized for their ability to accumulate in tumor but not in normal tissue. (See review by Vasir and Labhasetwar, 2005).

(2) The second goal is to amplify the signal. For this purpose, we will use a multivalent "guest" and a multivalent "host" substance that can form a hydrogel of increasing size at the tumor site. Whereas our original proposal was to use a pair of markers that can form a type of Schiff base, we also plan to study other complementary pairs of markers having greater binding specificity and avidity than the one originally proposed.

(3) The third goal is to detect the signal. For this purpose, we will use the "fluorescence resonance energy transfer" (FRET) approach, which takes advantage of the "dual probe" feature as described in step 2. Our detection capability will be further strengthened by working at the longest possible wavelengths, in the near infrared region of the spectrum.

The original SOW called for one chemist to initiate studies on the markers during the first year. Use of these markers for biological studies would begin in the second year. However, we have been moving forward in a balanced manner with both chemistry and biology personnel. As we enter the second year of the project, a series of conjugates are ready for biological testing.

BODY

Modification of the SOW: Tasks 1 and 2 of the SOW are concerned with synthesizing the "marker" substances that could recognize and bind to each other. We had planned to use these synthetic compounds in the first year for *in vitro* studies and in the second year for *in vivo* biological experiments. However, both the US ARMY MRMC and our own IACUC were concerned with some details of the animal protocol. Therefore, we had to spend more time than expected on preliminary biological aspects of the research program. This then caused a shift in the chemistry program. We have undertaken several *in vivo* experiments (see below) and have synthesized conjugates that pertain to the detection of the fluorescent probes as in Tasks 4 and 5, rather than the signal amplification process, as originally proposed in Tasks 1 and 2. We are pleased to report that we have made at least as much progress as planned even though the distribution of activities was different from the original proposal. For the sake of clarity, each aspect of the chemistry and biology work will be described separately. Chemistry work is mainly synthesis of novel reagents that will be used for the biology studies.

The concept.

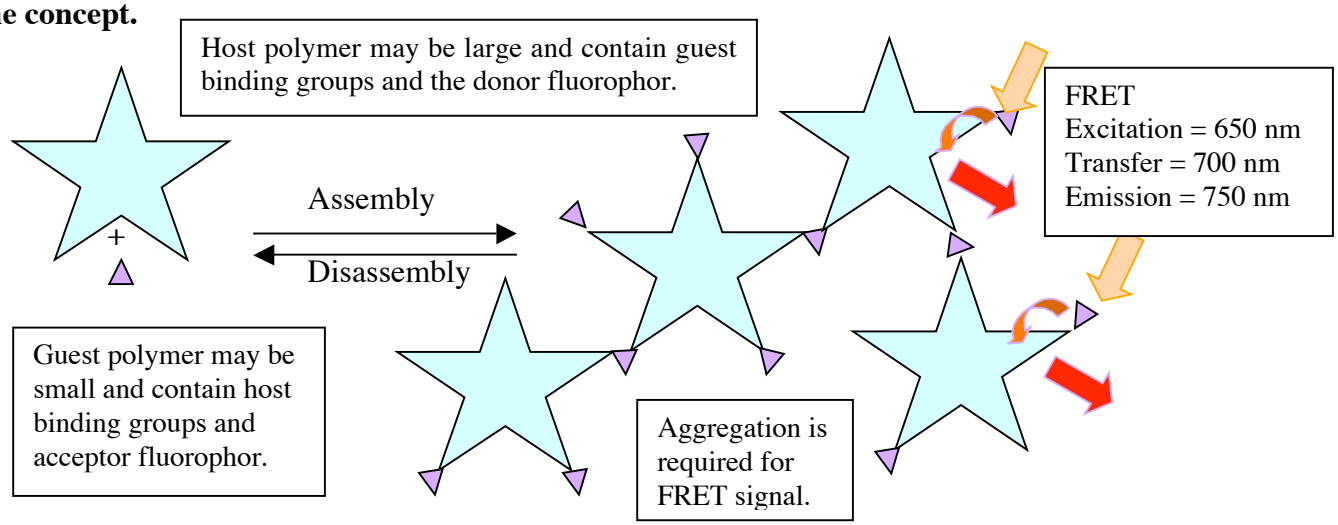
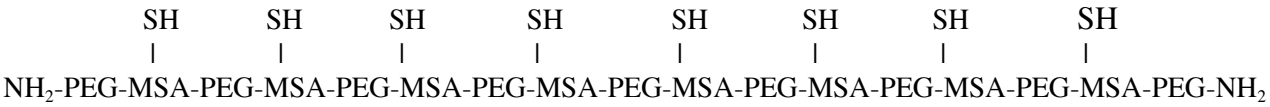


Figure 1. When the chemoselective probes (host and guest) bind to each other to form aggregates, a FRET signal is generated:

Synthesis of Polymers: The concept is to prepare two types of polymers, a "host" and a "guest" polymer, which will meet at a specific time only in tumors and not in normal tissues. These polymers will recognize each other (by chemoselectivity) and spontaneously form a microgel in the tumor. The host and guest polymers will each have a donor and an acceptor fluorophor for generating a FRET signal, but only when they come in close contact. Our gel-forming polymer is actually a copolymer of diamino-poly(ethylene glycol) and mercaptosuccinic acid:

Figure 2. Typical copolymer structure.



In figure 2, the size of the PEG polymer is 3,400 Da, and the 8 thiol- subunits along with the 9 PEG repeats give the conjugates a total weight of about 32,000 Da. Both the PEG and the copolymer are poly-disperse with regard to the number of repeats. Since the copolymer will be used as the carrier for tumor targeting and for the transition from a solution into a gel, we first had to establish quality control of its synthesis. The criteria for each batch are:
1. retention time on size exclusion chromatography (SEC)
2. time required for forming a gel under standard conditions.

We believe that the synthesis and quality control analysis of the copolymer has been standardized and we may now proceed with the use of this copolymer. This is confirmed by consistent size-exclusion chromatogram and time of gel formation with specific linkers. The copolymer was synthesized and the size distribution was analyzed by SEC (Figure 3). It can be seen that about one-third (by peak area) of the product is in a peak at 8.3 minutes, while the rest of the material in the reaction mixture is in the low molecular weight range (monomer, dimer trimer, etc.) and hence can easily be removed by thorough dialysis using a 50,000 Da cut-off filter (i.e. equivalent to a PEG MW of about 17,000 Da).

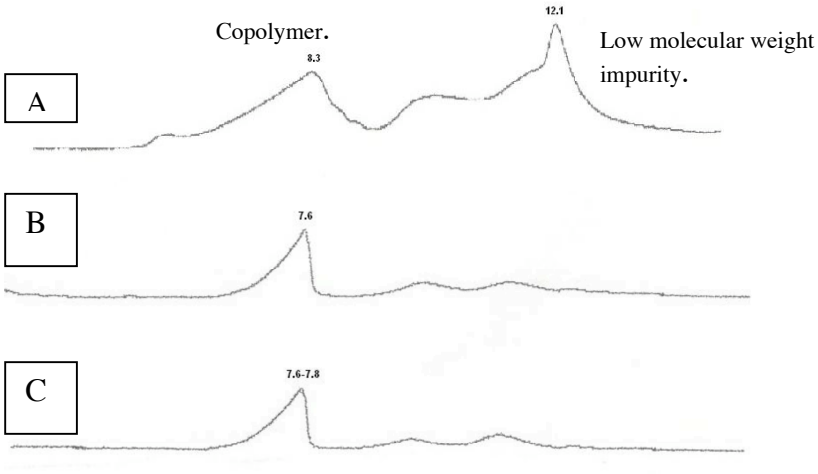


Figure 3. Size-exclusion chromatograms (60 cm x 0.78 cm Toso 3000SW column) of copolymer before dialysis (A) and after dialysis (B). C shows SEC profile for another copolymer after dialysis.

Table 1. Summary record of copolymer synthesis.

Batch	Yield (Trityl-on)	Yield after dialysis	Yield after Deprotection	Retention on SEC (min)*	Peak area on SEC (%total)**	Gelation Time (min)***
A.	2.1 g	1.7 g	1.1 g	7.6 min		<1, 3.6
B.	2.1 g	1.6 g	1.1 g	7.6 min		<1, 3.8
C.	2.1 g	1.4 g	0.81 g	7.6 min		<1, 3.8
D.	2.2 g	1.7 g	1.2 g	7.7 min		<1, 4.0

* Acceptable range is 7.5 – 8.0 minutes
**A cceptable range not determined yet,
***Time for gel formation is taken as the time solution ceases to flow (inverted tube method). Numbers are for two different cross-linkers. Acceptable range is 2 – 5 minutes using second cross-linker.

Four different batches of copolymer were synthesized using identical materials and conditions to ascertain the reproducibility of the synthetic procedure. The most important specification is gelation time, which is a critical factor in using the product. Thus, the time of gel formation is tested using two different cross-linking reagents. Each copolymer (24 mg) was dissolved in 480 μL of sodium phosphate buffer (pH 7.4). Cross-linker (2.2 mg) $BM(PEO)_3$ was dissolved separately in buffer (140 μL). Copolymer (400 μL) and cross-linker (100 μL) were mixed to give a 4% hydrogel. Gel timings are noted in Table 2. Since, the time for gel formation was too fast with $BM(PEO)_3$, another cross-linker HBVS (hexyl-bis-divinylsulfone) was employed. HBVS weight was 1.8 mg instead of 2.2 mg.

Conclusions. Copolymer synthesis is reproducible. Gelation rate is suitably rapid

Preparation of chemoselective polymers

It would be relevant to mention that the copolymers reported above are derived from Nathan et al. (1993) and had been prepared earlier in our laboratory by using N-α-t-Boc-L-aspartic acid (Pooyan et al. 2002) instead of tritylmercaptosuccinic acid (Qiu et al. 2003). The scheme of synthesis is shown

below and this leads to direct synthesis of copolymer with free amino functionality (Figure 4). Figure 5 depicts an alternative synthesis of the amino functionality via the thiol-containing PEG copolymer. The synthesis of aldehyde-containing polymer in this series of conjugates is shown in figure 6. The particular form of aldehyde group produced in this case is glyoxylic aldehyde, which is considered to be a strong Schiff base former. Figure 7 shows the synthesis of oxyamino group containing copolymer, which is favored for reaction with ketone/aldehyde groups to form Schiff bases. PEG conjugates will be used in a number of different ways (see below). Thus, the 2 favored chemoselective reactions are:

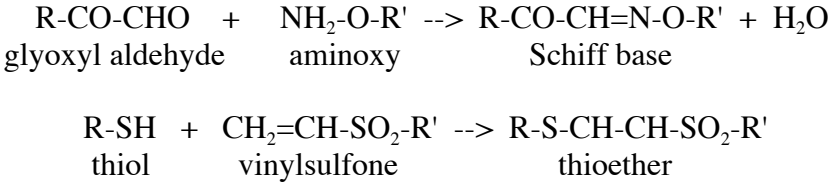
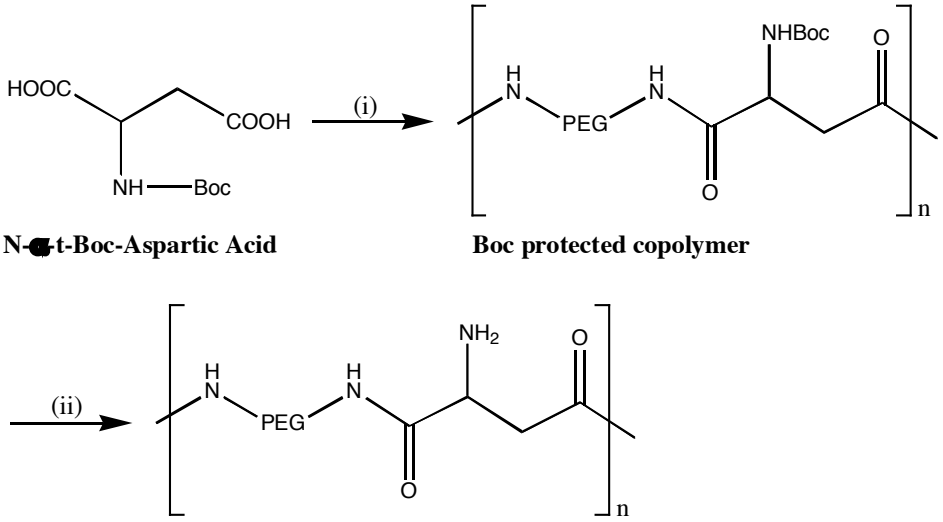


Figure 4.



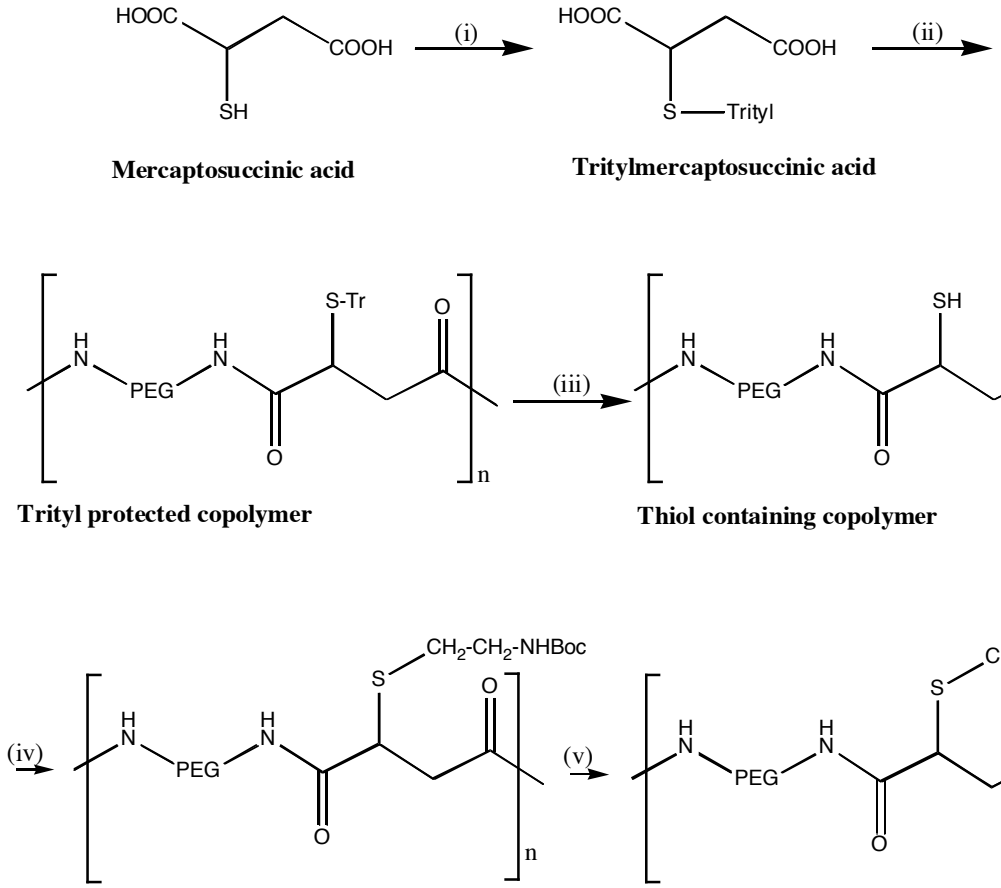
N-α-t-Boc-Aspartic Acid

Boc protected copolymer

Copolymer containing amine functionality

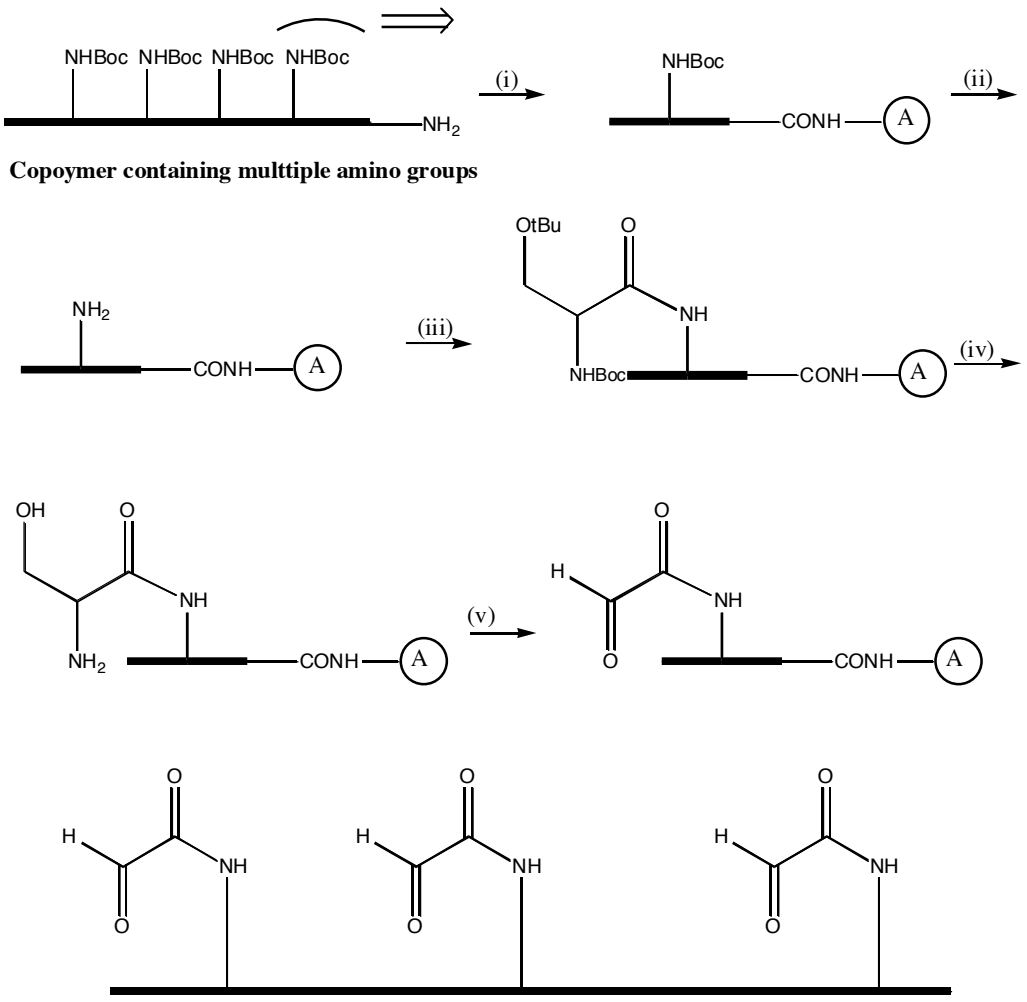
Synthesis of amine containing copolymer. (i) DiaminoPEG (MW = 3400 Da), N, N-diisopropyl carbodiimide, N, N-dimethylamino pyridine, p-toluenesulfonic acid monohydrate / Dichloromethane, r. t., 4d, dialysis; (ii) 50% Trifluoroacetic acid / Dichloromethane containing 5% triisopropyl silane and 5% water, r. t., 3h.

Figure 5.



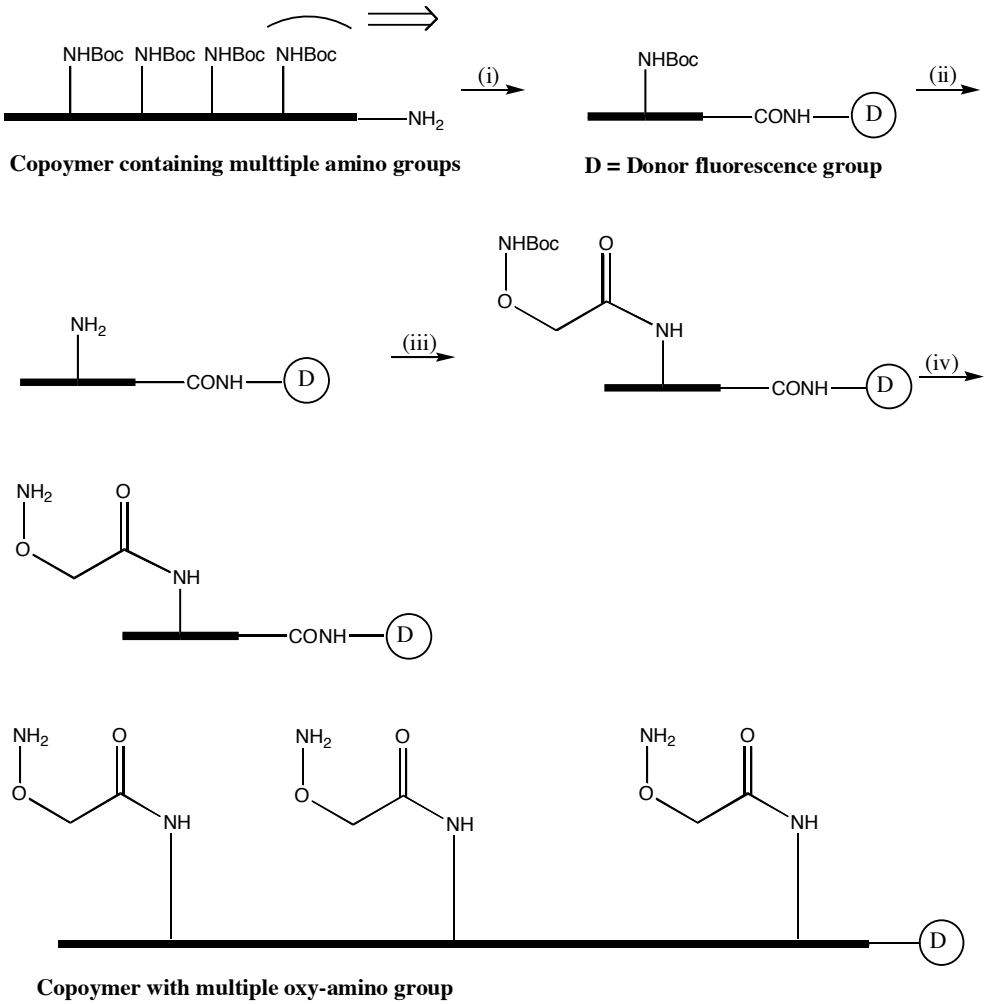
Synthesis of amine containing copolymer. (i) Trityl chloride / N, N-Dimethyl formamide, r. t., overnight; (ii) Diamino-PEG (MW = 3400 Da) / N, N-diisopropyl carbodiimide, N, N-dimethylamino pyridine, p-toluenesulfonic acid monohydrate / Dichloromethane, r. t., 4d, dialysis; (iii) Trifluoroacetic acid containing 5% thiioanisole, 3% ethanedithiol and 2% anisole, 3h; (iv) 2-(Bocamino) ethyl bromide; (v) TFA/DCM/TIS/H_2O.

Figure 6.



Preparation of copolymer with multiple glyoxylic aldehyde group. (i) Fluorophore-NHS / DIEA / DMF; (ii) 50% TFA in DCM containing 5% TIS and 5% water; (iii) Boc-Ser(tBu) / PyBOP / DIEA / DMF; (iv) TFA / DCM / TIS / H_2O; (v) $NaIO_4$ / H_2O.

Figure 7.



Preparation of copolymer with multiple glyoxylic aldehyde group. (i) Fluorophore-NHS / DIEA / DMF; (ii) 50% TFA in DCM containing 5% TIS and 5% water; (iii) Boc-NHOCH2CO-Succ / DIEA / DMF; (iv) TFA / DCM / TIS / H_2O.

PEG-conjugate synthesis:

We plan to test at least three sizes of PEG (selected from 3.4 kDa, 5kDa, 10kDa, 20 kDa, 30 kDa and 50kDa) for their pharmacokinetic properties. Therefore, we will synthesize these conjugates of PEG and HiLyte 750 (from Nektar and Anaspec, respectively) for "through the skin" measurement of the carrier injected as a subcutaneous depot to mimic a drug implant or to mimic targeting and accumulation of an imaging or therapeutic agent.

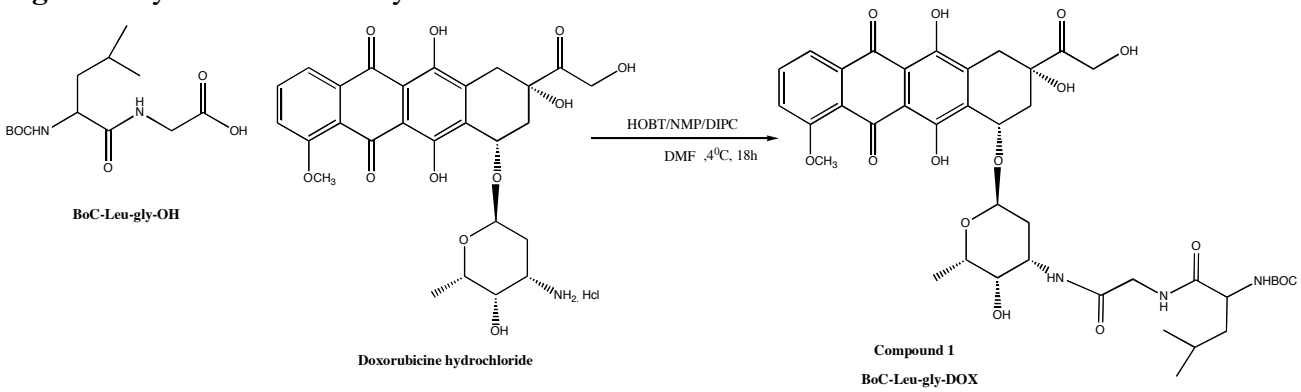
PEG_{MW}-activated ester + NH_2-HiLyte --> PEG_{MW}-HiLyte *for imaging*

PEG_{MW}-activated ester + NH_2-DOX --> PEG_{MW}-DOX *for therapy*

In the above reaction, MW is 5,000, 10,000 and 30,000 Da initially, but may be further optimized experimentally. Based on published data, placing a dipeptide such as Leu-Gly between the drug and

the carrier produces a superior prodrug, which is converted to active drug preferentially at the tumor site. The Leu-Gly-DOX will then be further PEGylated with three sizes of mPEG-SMB (5kDa, 10kDa and 30ka). We have optimized the following synthetic procedure for Leu-Gly-DOX. Boc-Leu-Gly-OH was coupled with DOX hydrochloride in different batch scales: 5mg, 50mg, 100mg and 200mg. The coupling reaction yielded 82% Boc-Leu-Gly-DOX product and the best reaction conditions are shown below (Figure 8):

Figure 8. Synthesis of Leu-Gly-DOX



The deprotection step for Boc was similarly optimized and required using 30% trifluoroacetic acid in dimethylformamide, and removal of the solvent without heating. Each synthetic intermediate and product is carefully analyzed for correct structure, typically mass spectrometry and NMR.

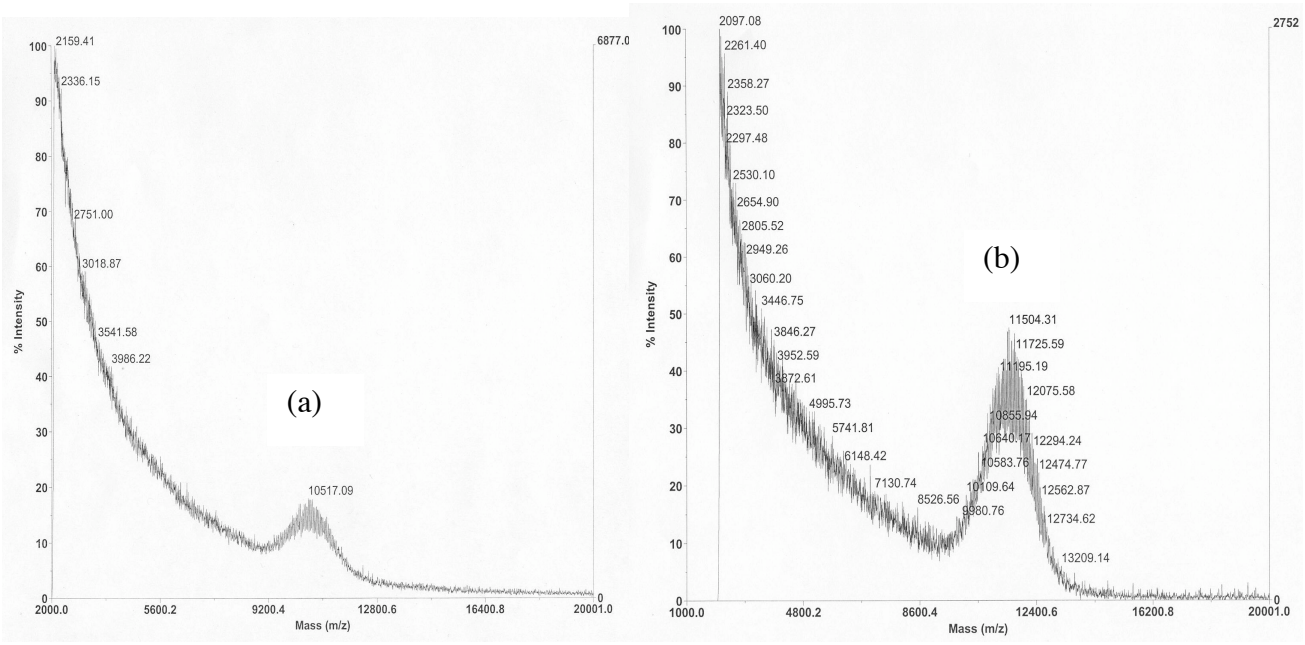
Similarly, PEG-HiLyte conugates were prepared, purified and characterized. An example of this quality control analysis is given in Figure 9. However, during their biological testing, we observed a problem with photobleaching of the fluorophor. Correction of this problem is in progress.

Mouse model of breast cancer:

One modification of the SOW concerned the animal model. We had proposed to use immunocompromised (nude) mice in order to test human breast cancer cell lines in a murine model. These nude mice are considerably more expensive to purchase and house, and more difficult to care for. Accordingly, we have decided to switch to the 4T1 model. Mouse 4T1 breast cancer cells provide a suitable experimental animal model for human mammary cancer. 4T1 cells can be easily transplanted into the mammary gland so that the primary tumor grows in the anatomically correct site. Moreover, as in human breast cancer, 4T1 metastatic disease develops spontaneously from the primary tumor. Also, the progressive spread of 4T1 metastases to the draining lymph nodes and other organs is very similar to that of human mammary cancer. However, we may still use a scaled-back nude mouse model to confirm anti-tumor results.

In our mouse model, (Figures 10-13), 4T1 cells in logarithmic growth phase were harvested and injected via subcutaneously into mammary gland of 8-week old BALB/c female mice (Jackson laboratories). Cells injected per mouse were 1.25×10^5, 2.5×10^5, 5×10^5 and 10^6, respectively. Animals were monitored daily for tumor onset, and tumor size was recorded daily after reaching measurable dimensions. Tumor size was either calculated by 0.5 x Length x width^2, or weighed after excision at the end point. Body weight was also monitored weekly. The experiment was terminated at 16 days after cell inoculation (Figure 13).

Figure 9.



Mass spectrum of (left) PEG10kDa-SMB (Before labeling: MW = 10,517 Da) and (right) PEG10kDa-Hilyte750 (After labeling: MW = 11,504). The product should be (10,517 + 1,071=) 11,588 Da, which is less than 1% different from the reaction product peak. PEG is polydisperse with regard to ethylene glycol subunits [$(CH_2\text{-}CH_2\text{-}O\text{-})_n$] and gives an envelope of ions differing by 44 Da

Notes:
(a) These labeling reactions were carried out by reacting excess (2 equivalents) amino dye (Hilyte Fluor™ 750 amine, MW=1,071Da) with 1 equivalent of PEG activated ester (PEG-SMB, MW = 5000Da, 10,000Da and 30,000Da) using 100mM phosphate buffer pH 8.0. The reactions were stirred overnight at room temperature. The crude PEG-Hilyte750 products were purified using Sephadex columns using 20mM phosphate buffer pH 8.0. The fractions containing the pure PEG-Hilyte750 were lyophilized to obtain a dry bluish green colored powder and characterized by MALDI-TOF/MS. (b) For purification of crude PEG5kDa-Hilyte750, a G-25 Sephadex column was used whereas for purifying crude PEG10kDa-Hilyte750 and PEG30kDa-Hilyte750, G-50 Sephadex columns were employed. In all the cases 20mM phosphate buffer pH 8.0 was used as the eluent.
(c) The synthesis of PEG-Leu-Gly-Doxorubicin using PEG activated esters (PEG-SMB, MWs =5000Da, 10,000Da and 30,000Da) and NH_2- Leu-Gly-Doxorubicin followed the same synthetic pattern as that of PEG-Hilyte750. Their purification technique was also similar to that of PEG-Hilyte750.

Conclusion: Six characterized conjugates (3 sizes PEG-Hilyte750 and 3 sizes PEG-Leu-Gly-Doxorubicin) are ready for biological testing.

Figure 10. An example of a BALB/c mouse bearing 4T1 tumor. Pulaski BA and Ostrand-Rosenberg S., *Current Protocols in Immunol*. (2000) *202*, 1-16.

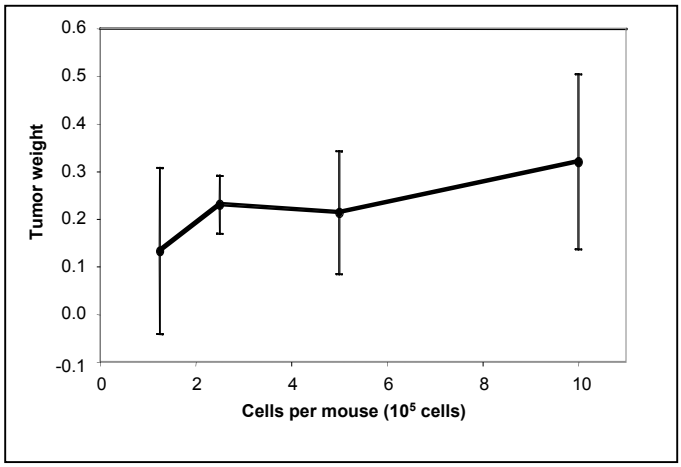


Figure 11. Tumor weight at end point (16 days after cell inoculation). At 16 days after tumor cell inoculation, mice were sacrificed with CO_2, and tumors were excised and weighted. Similar tumor weight was observed in all groups (n=3). (unpublished data)

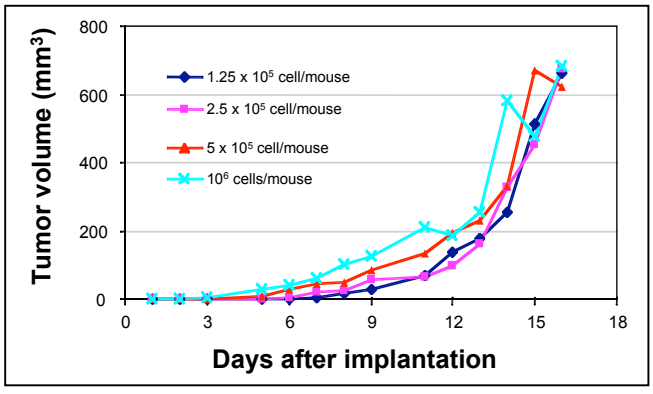


Figure 12. Time course of tumor development. Different groups displayed similar tumor volume. 10^6 cells/mouse group showed earlier tumor onset (n=3). (unpublished data)

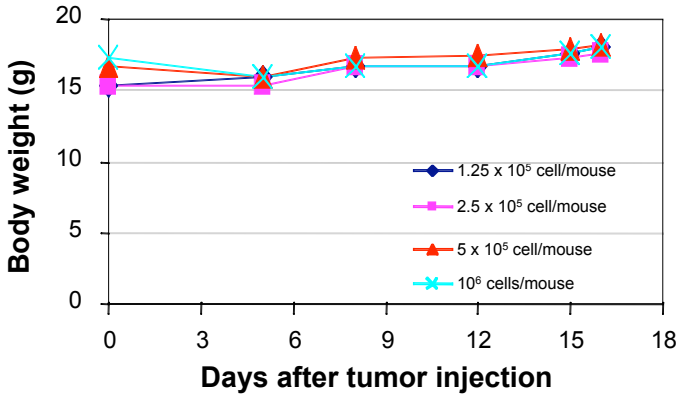


Figure 13. Effect of tumor growth on body weight. No wasting during 16 days of tumor growth. (unpublished data)

A pilot experiment was done in preparation for routine testing of anti-tumor activity in the mouse 4T1 model. In this study (Figure 14), mice were inoculated with 4T1 cells which appeared as tumors by day 9. Groups of 3 mice were treated on day 11 with Leu-Gly-DOX (low molecular weight prodrug) or PEG-Leu-Gly-DOX (high molecular weight prodrug) at a dose of either 1 or 10 mg/kg. The greatest tumor growth, as expected, was found with the uninjected control (Figure 14). The Leu-Gly-DOX prodrugs were more effective than their PEGylated prodrugs. Only one logistical problem was encountered. Removal of a 10% contamination of free DOX in the PEG-Leu-Gly-DOX sample caused a 2-day delay in treatment, at which time the tumors were getting too large.

We will avoid this problem by waiting for the synthesis and certification to be completed before initiating the inoculations. However, the mouse model appears to be ready for routine use.

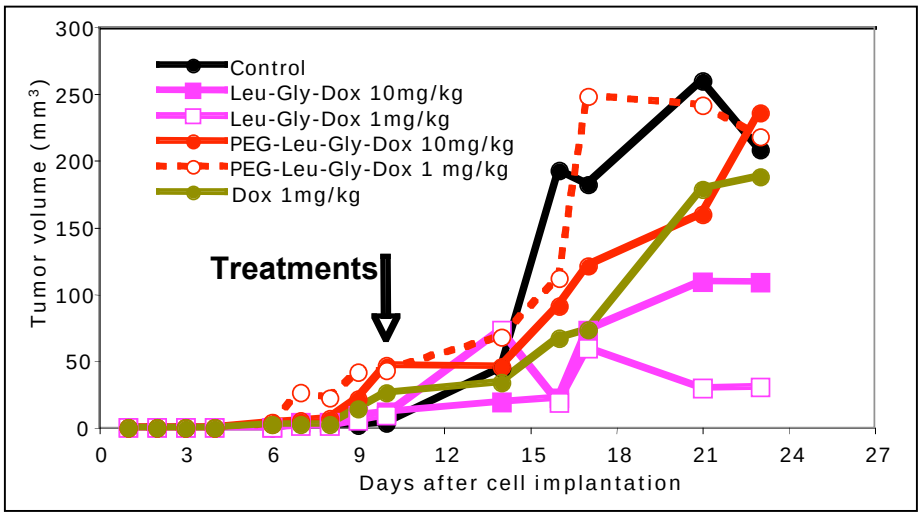


Figure 14. Pilot anti-tumor *in vivo* study. Each group started with 3 mice (n=3). This type of routine study will now be done more carefully and intensively. (unpublished data)

Skin Skan Detector: Calibration curve.

Hilyte Fluor 750 C2 maleimide (Anaspec) was dissolved in water at various concentrations. Twenty µl of each solution was added to a black 96-well plate. The fluorescence was measured at excitation wavelength of 754 nm and emission was monitored at wavelength of 778 nm. Concentration range up to 7.4 µM showed linearity (Figure 15). The lower limit of quantitation in the standard curve was at about 1 µmol/L or 1 nmol/mL or 1 pmol/µL, Thus, a 20 µL sample contains about 20 pmol, which is about 20 ng of fluorophor having a molecular weight of about 1,000 Da. We have determined that there is a 3-fold decrease in signal by measuring through mouse skin, and we have not yet measured the loss of signal through greater depths of breast or other tissues. Thus, in order to detect a signal that was attenuated 1000-fold going through tissue, at least 10 µg of fluorophor would have to be trapped in the tumor by EPR. These numbers appear to be reasonable.

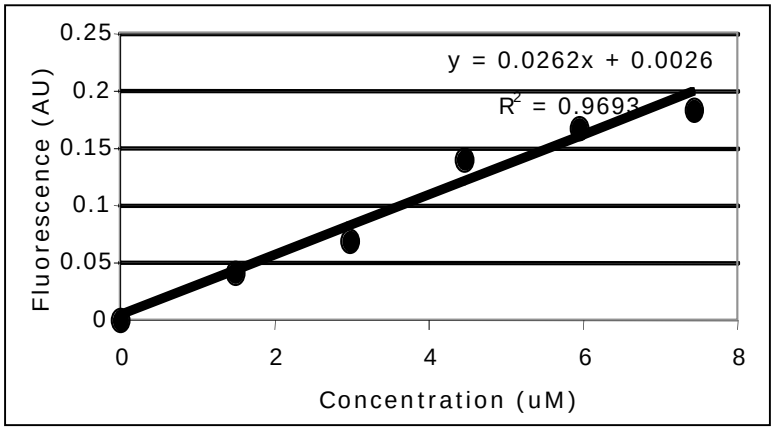


Figure 15. Dose-response of HiLyte 750 in wells. (unpublished data)

EPR effect in mice bearing tumor

This is a pilot experiment. BALB/c female mice were injected with 4T1 cells (12.5 x 10^5 cells/mouse) via sc into linguinal mammary gland. Seventeen days cell inoculation; mice were injected via i. v. with Hilyte conjugated PEG polymers (MW = 20 KDa and 50 KDa, respectively) dissolved in PBS at a dose of 20 µmol per mouse. PBS injected animal was used as control. Mice were shaved before measurement with Skin Skan at different time points. Non-tumor skin was taken as background and subtracted from readings at tumor site. Highest fluorescence was observed at about 24 h for both the 20 kDa and the 50 kDa polymers (Table 2). As predicted in the grant proposal, the seeding step is not sufficient for tumor detection. An amplification step, as proposed, will be needed to improve the tumor detection.

Table 2. Polymer content in tumor

Group	Tumor volume* (mm^3)	Polymer concentration** *in situ* (µM)	Polymer content in situ (nmol)	In situ/dose*** (%)
Control	115.1	0	0	0
20 KDa	228.0	0.70	0.16	0.0008
50 KDa	120.4	1.35	0.16	0.0008

*Tumor volume = length x $width^2$ x 0.5

** Concentration was based the above standard curve.

***Percentage = polymer content in situ/ injected dose x 100

KEY RESEARCH ACCOMPLISHMENTS

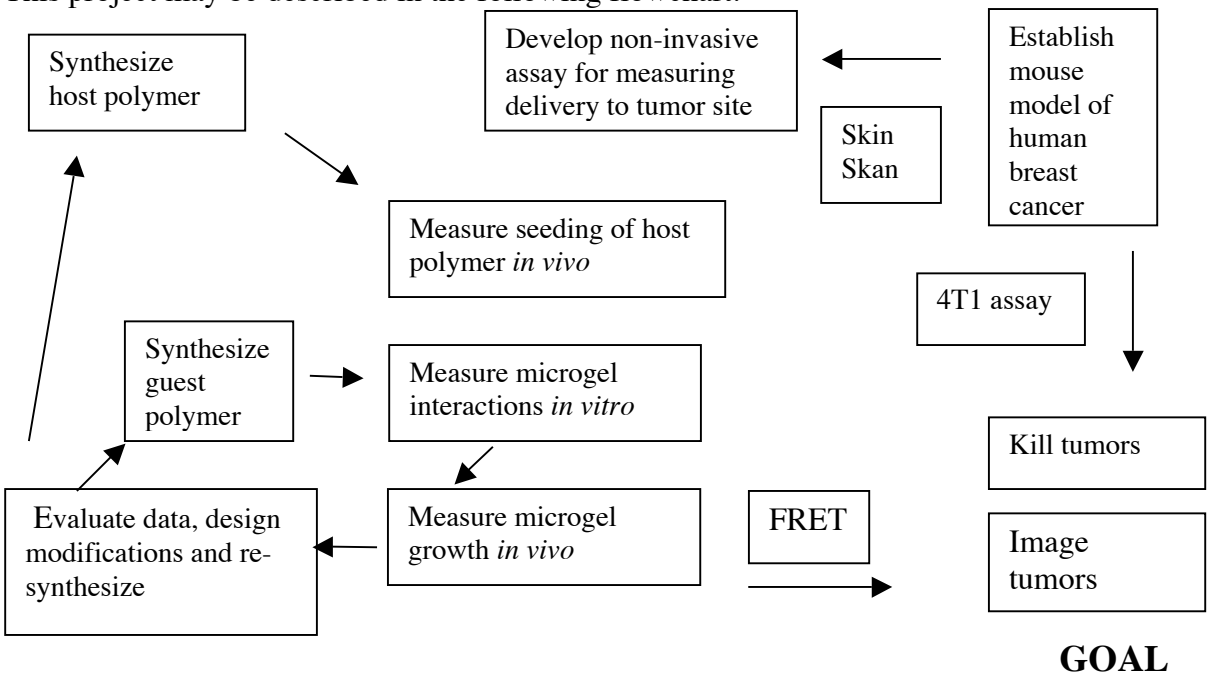
Nothing unexpected in this first year of designing polymers and establishing cancer models.

REPORTABLE OUTCOMES

(1) Whereas this project concerns imaging of breast cancer using EPR and FRET, we are also developing new formulations of anti-cancer drugs. From the few pilot experiments, we have shown a synergism of the efforts for the mutual benefit of both projects.
(2) In an separate project with a private company (West-ward Pharma Inc., Eatontown, New Jersey) to develop new dosage forms for traditional cancer drugs, the funding allowed us to purchase a Skin Skan fluorescence detector ($30,000, Spex, Edison, New Jersey), which is also available for use in this present project. The Skin Skan has dual monochromators for both the excitation and emission wavelengths. Our unit has been fitted with a red-sensitive photomultiplier tube for enhanced detection capability. The Skin Skan employs a bifurcated optical fiber for remote measurement on or through the skin. We will use this detector to quantitate the amount of an agent in an implanted hydrogel or the amount delivered to a targeted tumor. Indeed, an entire pharmacokinetic study can be done on each mouse, as opposed to the current practice of sacrificing one mouse for one time point. Owing to its outstanding optical features and computer software, the Skin Skan is ideal for the proposed studies, especially those using FRET.
(3) Another instrument is going to be procured soon by grants from West-ward Pharma Inc., New Jersey. This is the Waters Breeze size exclusion HPLC system (about $32,000). The system is equipped with dual wavelength absorption detector and differential refractometer with temperature control. The system is also being supplied with polymer standards in the molecular weight range of 400-100,000. The instrument will be available for use in the present project also and it will help us to determine the exact molecular weight of polymer sample being used in this work. This will also help us to ascertain the purity and stability of the polymer sample and pegylated prodrugs with greater precision. Besides, it may be used to develop assay for various pegylated drugs in biological samples.
(4) We have established in our laboratory a syngeneic mouse model of breast cancer that is considered to be the most relevant to the human disease.
(5) We have established in our laboratory approaches and techniques for synthesizing and analyzing PEGylated prodrugs.

CONCLUSION

This project may be described in the following flowchart:



The overall plan remains the same, but more attention than originally planned has been placed on biology in this first year. Based on pilot experiments, all systems are ready to go.

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