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NOVEL MOLECULAR PROBES FOR ^{19}F MAGNETIC RESONANCE IMAGING: SYNTHESIS & CHARACTERIZATION OF FLUORINATED POLYMERS

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Abstract: Fluorine labelled polymers have been synthesized and characterized to investigate vascular phenomena *in vivo* with ^{19}F Magnetic Resonance Imaging (MRI). The polymers have molecular weights in the range 10 k to 98 k and include polylysines and functionalized dextrans (polyamino dextrans and polyaldehyde dextrans). These fluorinated polymers exhibit a single sharp ^{19}F signal, appropriate ^{19}F MR detection sensitivity, and the biocompatibility necessary for *in vivo* MRI.

Cancer is the second most common cause of death in the United States. The cure of cancer is heavily dependent on early detection and the effective implementation of therapy[1]. It is known that tumor microvessels are hyperpermeable to macromolecules (polymers)[2] and this phenomenon could be exploited in diagnosis and therapy. It has been suggested that drugs may be selectively targeted to tumors by conjugation with polymeric carrier molecules. In addition, polymers provide multiple substitution sites and are thus ideally suited to the dual purpose of anchoring and targeting sensor molecules.

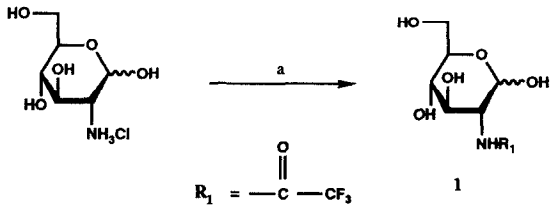
Magnetic Resonance Imaging (MRI) and spectroscopy provide non-invasive methods of observing tissue *in vivo*[3]. Proton MRI provides three-dimensional map of tissue structure with high spatial resolution. Previous studies in this laboratory using proton MRI have shown that a polymer-bound derivative of the relaxation agent gadolinium-DTPA selectively localized in the well-perfused regions of tumors[4]. However, quantitative measurements by contrast may be difficult. A ^{19}F MR image correlated to a proton image offers several advantages: no background fluorine signal from soft tissues, high sensitivity, ease of implementation using appropriate MR techniques[5] and a quantitative means of describing vascular properties through signal intensity.

We are developing both MR techniques and fluorine-labelled molecular probes to investigate vascular phenomena *in vivo*. Previous ^{19}F MR investigations of tissue perfusion and vascular density have exploited small monomeric materials such as trifluoromethylsulfonic acid[6]. These are subject to very rapid renal clearance. The distribution of perfluorocarban emulsion in a tumor has been used to examine perfusion defects following therapy[7]. However these nanoparticles are subject to extensive reticuloendothelial uptake and excessive tissue retention[5]. We have now synthesized and characterized a variety of fluorine-labelled polymers to produce images of tumors *in vivo* by ^{19}F MRI. We envisage the use of polymeric markers to reveal hyperpermeable structures e.g., fenestrated endothelium of a tumor vascular bed, on the basis of accumulation of fluorinated polymeric material in tissue.

The extravasation of polymers in tissues is governed by a number of transport factors, such as molecular weight, hydrophilicity, charge, and shape[8]. Based on these properties, polylysines and functionalized dextrans (polyamino dextrans and polyaldehyde dextrans) were chosen as carrier molecules for conjugating the fluorine markers. Several synthetic methods have been employed to conjugate fluorine markers to these carrier molecules.

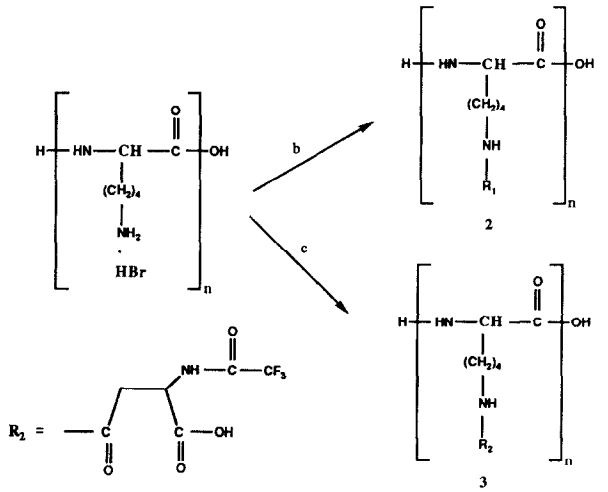
The reaction conditions have been optimized using various reagents in order to achieve high degree of incorporation of fluorinated moieties into these carrier molecules.

The reactions were initially evaluated and established using a monomeric unit, 2-amino-2-deoxy-D-glucose (Sigma, MO). The amino group was selectively trifluoroacetylated using S-ethyltrifluorothioacetate (SETFA)[9,10] to produce 2-deoxy-2-(trifluoroacetamido)-D-glucose(TFAG, **1**) (Scheme 1). **1** has been employed as a prototype molecule to determine relative ^{19}F MR sensitivity of fluorinated polymers and develop ^{19}F MRI *in vivo*.



Scheme 1. a) Methanol/MeoNa, $EtSCOCF_3$

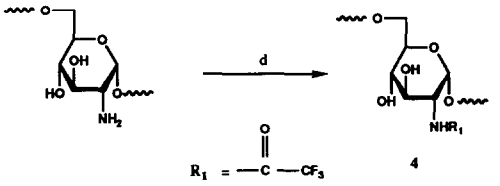
Polylysines are cationic in nature and shown to have high affinity for tumors[11]. Fluorine derivatives of polylysines have been prepared to examine the effect of charge on permeability. Trifluoroacetylation of polylysine (98 k ; Sigma, MO) using SETFA yielded a water insoluble fluorinated polymer[12] **2** (Scheme 2) which had good solubility in methanol. A water soluble fluorinated derivative of polylysine was produced by derivatizing polylysine (98 k) with trifluoroacetamidosuccinic anhydride, resulting in the formation[13] of **3** (Scheme 2). The additional free carboxylic groups in **3** may alter the cationic nature of polylysine and enhance water solubility. The extensive functionality of polylysines facilitated efficient derivatization and yielded the product with good ^{19}F MR sensitivity (Table I); however, polylysines exhibit considerable cytotoxicity which limits their utility.



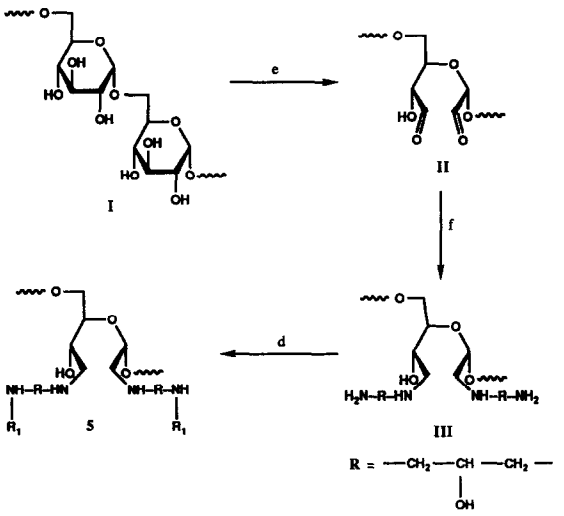
Scheme 2. b) Phosphate Buffer, $EtSCOCF_3$; c) 0.1 M NaHCO3, Trifluoroacetamidosuccinic anhydride

By contrast dextrans and functionalized dextrans have been shown to be useful in drug delivery. They have desirable properties such as water solubility, pH stability, low toxicity, and increased plasma persistence of conjugated moiety[14] to use as carrier macromolecules. The derivatization of dextran yields ester linkages which are labile and susceptible to hydrolysis. This can produce chemical shift artifacts and can cause considerable toxicity. However, functionalized dextrans (polyamino dextran and polyaldehyde dextran) on derivatization yield fluorinated products with amide or alkyl linkages respectively, which are far more stable than ester linkages.

Selective trifluoroacetylation of amino groups in polyamino dextran (10 k, 40 k, 70 k; Molecular Probes, OR) was achieved[15] using SETFA (Scheme 3). Trifluoroacetylated polyamino dextran (**4**), obtained by derivatization of commercial polyamino dextran, had poor ^{19}F MR sensitivity. This was attributed to the small number of amino groups available for derivatization in commercially available polyamino dextrans (viz: 40 k, 13 amine equivalents/mole). In order to introduce additional amino groups in the polymeric backbone, we synthesized polyamino dextran starting from dextran. Dextran (**I**) was partially oxidized with sodium periodate to form polyaldehyde dextran (**II**), which was reacted with 1,3-diamino-2-hydroxypropane to generate the Schiff base. The Schiff base was reduced with sodium borohydride to give the desired polyamino dextran[16] (III) (Scheme 4). The synthesized polyamino dextran (**III**) had additional amino groups available for derivatization and the trifluoroacetylated derivative **5** (Scheme 4) showed enhanced ^{19}F MR sensitivity (Table I).

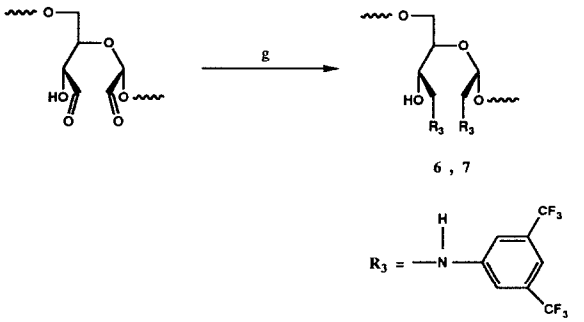


Scheme 3. d) Formamide/pyridine, $EtSCOCF_3$



Scheme 4. e) Water, $NaIO_4$; f) $H_2NCH_2CHOHCH_2NH_2$, $NaBH_4$

Fluorinated polyaldehyde dextran has been prepared by reaction of polyaldehyde dextran (PAD, 40 k; Pharmachem, PA) with 3,5-bis(trifluoromethyl)aniline to form an imine, which on subsequent reduction with $NaBH_4$ yielded the fluorinated derivative[17] **6** (Scheme 5). The product **6** obtained by derivatization of commercial PAD, resulted in a poor degree of derivatization and poor yields. This was due to the presence of too many aldehyde groups in commercial PAD (138 CHO/mole dextran) which decreased its solubility in any solvent (water/organic solvent) and does not allow efficient derivatization. In order to overcome the problem of low solubility encountered with commercial PAD, polyaldehyde dextran (II) synthesized by partial oxidation of dextran (Scheme 4) was derivatized under the reaction conditions described above to obtain **7** (Scheme 5). Synthesized PAD (II) had good solubility in water as well as formamide, thus leading to more efficient derivatization. Hence, **7** was obtained in good yields and had better ^{19}F MR sensitivity (Table I).



Scheme 5. g) Formamide/Pyridine, $H_2NC_6H_4(CF_3)_2$, $NaBH_4$

All these polymers have been obtained in good yields and characterized by ^{19}F NMR, I.R., and elemental analyses. They exhibit a single sharp ^{19}F NMR signal and some of them have appropriate ^{19}F MR detection sensitivity (Table I) and biocompatibility[18] necessary for *in vivo* MRI.

Purification and Characterization of Polymers:

The polymers **3** to **7** were purified by exhaustive dialysis and isolated in good yields (Table I). To evaluate the product purity and integrity of the polymers following the reaction, gel permeation chromatography (GPC) was performed using two Waters ultrahydrogel columns (500 & 250 Å) at 35 ^0C. Sodium nitrate (0.1 M $NaNO_3$; for dextrans) was used for elution at a flow rate of 0.8 ml/min and 5% ammonium dihydrogenphosphate adjusted to pH-4.0 (using phosphoric acid) + 3% ACN for polylysines. A Waters 410 differential refractometer was used for detection. Analyses of fluorinated polymers showed a smooth GPC profile without any formation of additional low or high molecular weight products.

^{19}F NMR: Spectroscopy

Trifluoroacetylglucosamine (**1**) exhibits 2 resonances separated by 0.3 ppm resulting from the α and ß anomeric forms. *In vivo* the typical line width is such that these are unresolved and do not produce chemical shift artifacts in ^{19}F MRI. The derivatized polymers **2** to **7** exhibit a single sharp ^{19}F signal, and thus will not produce any chemical shift artifacts in ^{19}F images. We have determined relative ^{19}F MR sensitivity of fluorinated polymers compared to TFAG (**1**). In other words, the relative sensitivity is defined as the number of mgs of

fluorinated polymer needed to give the same intensity of ^{19}F NMR signal as that obtained with 1 mg of TFAG (**1**). Some of these polymers have ^{19}F sensitivities approaching that of TFAG (**1**) (Table I).

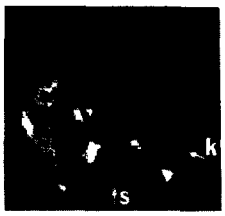
Table I: Characteristics of Fluorinated Polymers

POLYMERS SYNTHESIZED	SOLUBILITY	^{19}F NMR δ ppm[a]	^{19}F NMR SENSITIVITY	YIELD[b]
1	Water	0.22 -0.10	2 mg/mg NaTFA	70%
2	Methanol	0.00	---------	80%
3	Water	0.00	1 mg/mg TFAG	85%
4[c]	Water	0.00	>50 mg/mg TFAG	75%
5[d]	Water	0.00	5 mg/mg TFAG	70%
6[c]	Water	12.70	33 mg/mg TFAG	35%
7[d]	Water	12.70	10 mg/mg TFAG	76%

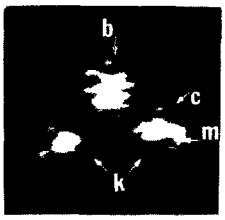
a Chemical shift w.r.t. TFA, b Based on amount (mg) of polymer recovered,
c Commercial polymers used, d Functionalized polymers used

***In Vivo* Imaging:**

We have developed ^{19}F MRI techniques using the prototype molecule, TFAG (**1**). This has a ^{19}F spin-lattice relaxation time $T_1 \sim 1$ s and spin-spin relaxation time $T_2 \sim 900$ ms at 4.7 T *in vivo*. Following an intravenous (iv) injection of 300 mg TFAG (**1**) into a tail vein of a mouse (25 g), ^{19}F image data sets were obtained in 12 minutes. Within 30 minutes of injection correlated ^1H and ^{19}F MRI showed intense signal from the kidneys (Figure 1). Over 3 hours this signal diminished with concomitant increase in signal from the bladder. The monomer TFAG (**1**) is valuable in the study of the vasculature at short times.



k - kidney
s - spine
b - bladder
c - cortex
m - medulla



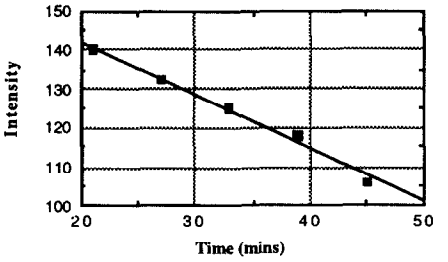
^1H Image **FIGURE 1** **^{19}F Image**

***In Vivo* Spectroscopy:**

We studied the clearance of TFAG (**1**) from Meth-A tumor in mouse (25 g) following iv administration in the tail vein (150 mg). Observations using a surface coil started 15 min after infusion. Within the first 15 min the concentration in tumor had already reached maximum and the clearance of the agent from the tumor as measured by the decrease in the intensity of ^{19}F signal is shown below (Graph 1).

GRAPH 1

CLEARANCE OF TFAG FROM METH A TUMOR



We have obtained ^{19}F images of the polymers *in vitro* and are currently assessing their utility *in vivo*.

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12. Trifluoroacetylation of polylysine: Polylysine (98 k) was dissolved in phosphate buffer and 5 fold molar excess of SETFA was added dropwise with stirring for 30 min. Solution was allowed to stir for 2 h and the solid separated out was isolated by centrifugation, washed with water to obtain **2**.
13. Trifluoroacetamidosuccinylation of polylysine: A 10-fold molar excess of trifluoroacetamidosuccinic anhydride was added to 0.1 M $NaHCO_3$ solution of polylysine (98 k) while maintaining the pH-8.0 by adding 0.1 M $NaHCO_3$ solution. It was stirred overnight. The mixture was exhaustively dialyzed against water and lyophilization yielded the fluorinated polylysine (**3**).
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15. Trifluoroacetylation of polyamino dextran: Polyamino dextran was dissolved in formamide/pyridine (8 : 2). 10 fold molar excess of SETFA was added dropwise over 30 min and stirred overnight. Solution was poured onto chilled absolute ethanol and maintained at -10 ^0C till gelatinous ppt appears. Solid obtained by centrifugation was washed with cold ethanol and subjected to exhaustive dialysis. Lyophilization yielded **4**.
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17. Fluorination of polyaldehyde dextran: Polyaldehyde dextran (40 k) was dissolved in formamide/ pyridine (9 : 1). 3,5-Bis(trifluoromethyl)aniline was added and stirred overnight. $NaBH_4$ was added to the mixture and stirred at 37 ^0C for 2 h. To cooled solution, acetone was added to precipitate the solid and left in refrigerator. The solid was obtained by centrifugation and washed with cold acetone followed by anhydrous ether. The product was dissolved in deionized water and exhaustively dialyzed against water. The solution was lyophilized to yield **6**.
18. Fluorinated glucosamine (**1**), polyamino dextrans (**4 & 5**), and polyaldehyde dextrans (**6 & 7**) could be injected in mice in high doses (up to 50 -150 mg) without any apparent acute toxicity. However fluorinated polylysine (**3**) was highly toxic even in low doses (<10 mg).