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FLUORINATED MACROMOLECULAR PROBES FOR NON-INVASIVE ASSESSMENT OF pH BY MAGNETIC RESONANCE SPECTROSCOPY

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Abstract: Fluorinated polymeric molecular probes have been synthesized and characterized for non invasive assessment of pH with ^{19}F Magnetic Resonance Spectroscopy (^{19}F MRS). 3-Fluorosalicyl-aldehyde (3-FSA) has been employed as a prototype ^{19}F pH indicator to conjugate to carrier molecules (polyamino dextran, polylysine and albumin). These 3-FSA polymer conjugates exhibit a single sharp ^{19}F signal, moderate ^{19}F pH sensitivity, and the physiological pKa necessary for *in vivo* measurement of pH by ^{19}F MRS.

Intracellular and interstitial pH play an important role in cell function, growth, and development[1]. It has been shown that tumor pH influences cell thermosensitivity, radiation sensitivity, and proliferation[2]. Thus, an accurate non-invasive assessment of tissue pH may provide a prediction of therapeutic efficacy. pH measurement by NMR has several advantages: continuous nondestructive monitoring of pH in cell suspensions, tissues or perfused organs; spatial resolution; determining simultaneously the pHs of several compartments within cells and a relatively non-invasive procedure.

^{19}F NMR spectroscopy is a particularly attractive technique for the detection, quantitation, and structure elucidation of ^{19}F-containing compounds in tissues and biological fluids *in vitro*. It has gained interest as a non-invasive method for studying various physiological parameters and drug metabolism *in vivo* and as a basis for NMR imaging. The specific advantages of ^{19}F are: 1) low intrinsic concentration in soft tissues of the body; 2) high nuclear magnetic resonance sensitivity, and 3) a magnetogyric ratio close to that of ^{1}H, facilitating observation of ^{19}F with standard RF components.

The NMR characteristics of the fluorine nucleus are particularly sensitive to the microenvironmental milieu. Thus, we and others have used ^{19}F NMR to determine such diverse parameters as pO_2[3], temperature[4], membrane potential[5], pH[6], cation concentrations[7] and perfusion[8]. NMR techniques for measuring cellular ion concentrations offer the opportunity for spatial and temporal resolution of indicator signal. Fluorinated NMR indicators offer potential advantages, including the ability to perform studies in cells despite significant fluorescent background (e.g., erythrocytes), the ability to study perfused organs, and greater selectivity of NMR observations for different and potentially interfering ions. In our previous communication[9], we demonstrated the ability to label polymers with fluorine for ^{19}F MRI/MRS applications. We now show the functionalization of polymers with fluorine to create fluorinated macromolecular probes for the assessment of physiological functions (e.g., pH) using ^{19}F MRS/MRI.

Traditional techniques of pH measurement are invasive (microelectrodes), or limited to surface tissue (fluorescence). NMR offers a non-invasive alternative and ^{31}P NMR has been applied extensively to the measurement of intracellular pH[10]. Measurements of pH using the chemical shift of Pi (inorganic phosphate) require caution because the pKa is affected by ionic strength, protein solvation, and temperature[11]. This method uses the chemical shift difference between Pi and PCr (phosphocreatine), but well perfused tissues often lacks Pi, whilst hypoxic tissues may be poor in PCr. The Pi signal may also be masked by intense phosphodiester signals (e.g., neo-natal brain) and the cellular pH may extend beyond the effective pH measurement range of Pi under severe hypoxia[12]. pH determinations using ^{1}H NMR of metabolites have been reported by spin echo technique[13]. The internal pH of the cell was estimated from the chemical shift of the histidine C(2) resonances of haemoglobin. ^{1}H NMR suffers severely from the intense water signal and multiple overlapping metabolite resonances. ^{19}F NMR indicators have been proposed previously, however some of the reported molecules were poorly sensitive to changes in pH[6] and others lacked the appropriate pKa or could not be infused into cells[14].

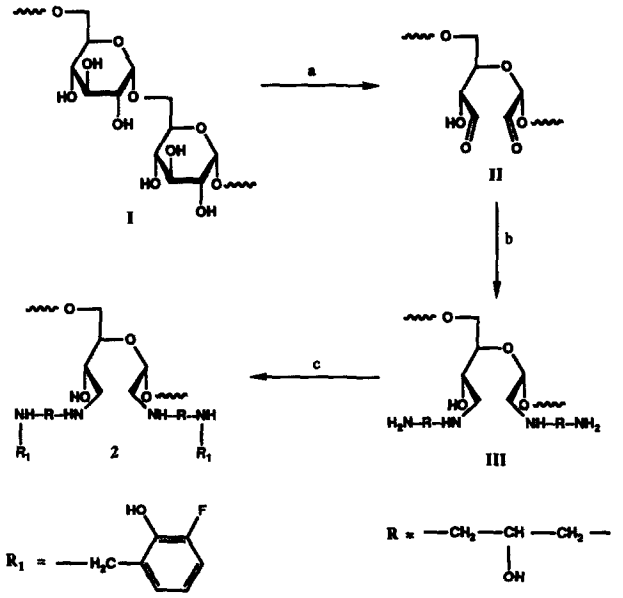
Our goal is to develop fluorinated molecular probes for non-invasive, *in vivo* measurement of pH using ^{19}F MRS/MRI. As any sensing molecule administered *in vivo* would quickly distribute to different biological compartments, the ability to measure pH would be impaired if the molecule were able to freely and rapidly move between compartments or were altered by biological processes. We are investigating the feasibility of attaching pH indicators to carrier polymers without loss in pH sensing capabilities or ^{19}F signal strength as a means of targeting and for preventing such movement. Additionally, the biological toxicity of the pH marker may be masked/altered upon coupling to non-toxic carrier polymers. We envisage the use of pH indicator polymer conjugate to enhance vascular retention and facilitating investigations of tumor vascular and interstitial pH.

Macromolecules such as albumins, globulins, dextrans and synthetic polymers (e.g., polylysine) accumulate in tumor tissues because these tissues have a vascular network characterized by both enhanced permeability of the neovasculature and a lack of lymphatic recovery system[15]. Based on these properties, functionalized dextrans (polyamino dextran), polylysine and proteins (albumin) were chosen as carrier molecules for conjugating the pH indicator. We have developed and tested several pH sensitive ^{19}F NMR indicators, primarily fluorophenol structures. We have employed 3-fluorosalicylaldehyde (3-FSA, Aldrich, WI; **1**) as a prototype molecule to conjugate to transport polymers and studied the pH properties of polymer-3-FSA conjugates.

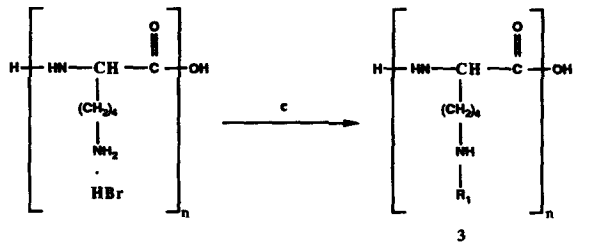
Dextrans and functionalized dextrans of various molecular weights (40 k to 110 k) have been used for many years as plasma expanders[16]. In addition, dextrans have been proposed as carriers for drugs[16], radiolabeled markers, and ^{1}H NMR contrast agents[17], by virtue of the following properties: 1) well-defined and repetitive structure, 2) high water solubility, 3) high stability, 4) availability of numerous reactive functional groups, 5) availability of different molecular weights, 6) low pharmacological activity and toxicity, 7) protection of conjugated moiety from biodegradation. Initial attempts to prepare trifluoroacetylated derivatives of commercial polyamino dextrans resulted in poor ^{19}F MR sensitivity[9]. We overcame the problem of the small number of amino groups available for derivatization in commercial polyamino dextran by introducing additional amino groups in the polymeric backbone. Dextran (40k; Sigma, MO; **I**) was partially oxidized with $NaIO_4$ to form polyaldehyde dextran (**II**), which was reacted

with diamine to generate the Schiff base. The Schiff base was reduced with sodium borohydride to produce polyamino dextran[18] (III) (Scheme 1). Reaction of 3-FSA with polyamino dextran (III) yielded a Schiff base, which was reduced with $NaBH_4$. Dialysis and lyophilization yielded dextran bound 3-FSA[19] (2) (Scheme 1).

The cationic polylysines have high affinity for tumors cells[20]. Polylysine-3-FSA conjugate has been prepared by reaction of polylysine (98 k; Sigma, MO) with 3-FSA to generate an imine, followed by reduction with $NaBH_4$ to produce the conjugate 3 (Scheme 2).



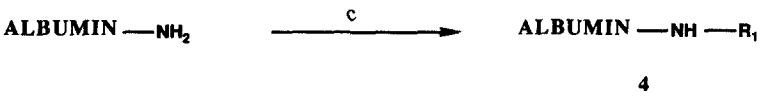
Scheme 1. a) Water, $NaIO_4$; b) $H_2NCH_2CHOHCH_2NH_2$; $NaBH_4$; c) 3-Fluorosalicylaldehyde, 0.5% K_2CO_3; $NaBH_4$



Scheme 2.

Albumin has been investigated as a potential carrier of anticancer drugs[16], radiolabeled markers and ^1H NMR contrast agents[21] for the following reasons: 1) ready availability as a pure and uniform compound, 2) good biological stability, 3) defined and appropriate molecular weight, 4) ease of chemical

substitution, 5) low toxicity, 6) lack of antigenicity of "homologous" albumin, and 7) well characterized cellular interactions and pharmacokinetics. Albumin-3-FSA conjugate was synthesized by reaction of Bovine Serum Albumin (Sigma, MO) with 3-FSA to form an imine, which on subsequent reduction with $NaBH_4$ yielded the fluorinated derivative **4** (Scheme 3).



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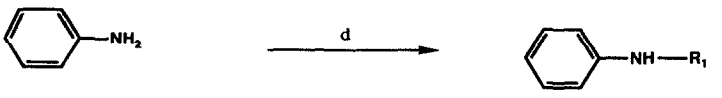
Scheme 3.

In developing macromolecular pH indicators, it is however necessary to evaluate the possible changes in pH sensing characteristics of the indicator molecule (3-FSA) upon coupling to the polymer, i.e., the effect of large molecular size, electric charge, shape and lypophilicity/hydrophilicity. To this end we coupled 3-FSA to a small molecule (monomer, specifically aniline) using linkages similar to those used for polymers, and studied the pH properties of the product[22] (**5**; Scheme 4). The pH response obtained for **5** was similar to that of **2** to **4** (Table I). These studies suggest that polymers do not exert any strong effect on the pH sensing capability of 3-FSA as compared with monomer.



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Scheme 4. d) 3-Fluorosalicylaldehyde, CH_3OH; $NaBH_4$

Purification and Characterization of Polymers

The polymer-3-FSA conjugates (**2 to 4**) were purified by exhaustive dialysis and characterized by ^{19}F NMR, I.R., and elemental analyses. Gel permeation chromatography (GPC) was performed using two Waters ultrahydrogel columns (500 & 250 Å) at 35 ^{0}C to determine the product purity and integrity of the polymers. Sodium nitrate (0.1 M $NaNO_3$; for polyaminodextran) was used for elution[23] at a flow rate of 0.8 ml/min with Waters 484 (UV/VIS) and 410 differential refractometer. The polymer-3-FSA conjugates showed a smooth GPC profile similar to that of starting polymers, excluding the possibility of formation of high or low molecular weight products.

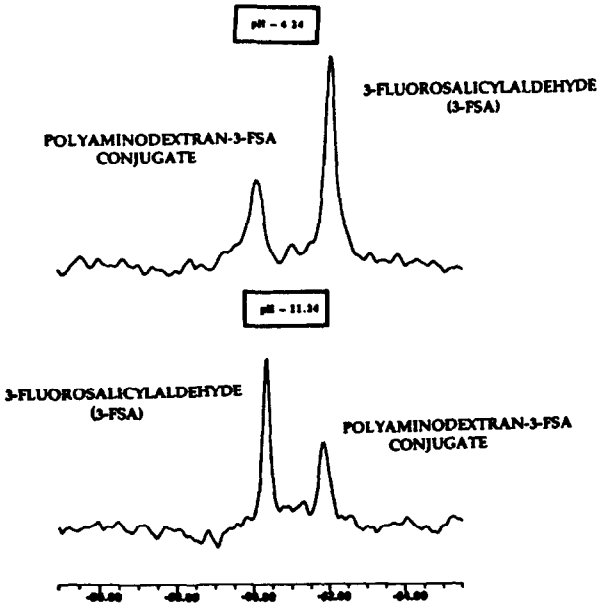
^{19}F NMR Spectroscopy

The polymer-3-FSA conjugates (**2** to **4**) exhibit a single sharp ^{19}F signal, and thus, will not produce any chemical shift artifacts in ^{19}F MRI/MRS. We have determined the chemical shift sensitivity and pKa using ^{19}F MRS over pH range 2.0 to 10.0 for 3-FSA (**1**) and 3-FSA conjugates (**2** to **5**) (Figure 1 & 2 and Table I). The polymer-3-FSA conjugates (**2** to **4**) have chemical shift sensitivity of 1.36 - 1.41 ppm over pH range of 5.5 - 8.5 and appropriate physiological pKa (~7.0 - 7.2)[24] and these are similar to 3-FSA(1). Interestingly, the change in chemical shift is reversed in all the conjugates (both polymeric and monomeric) compared with 3-FSA alone (Figure 1 & 2 and Table I).

^{19}F NMR Spectroscopy

FIGURE 1

Mixture of 3-FSA (1) & Conjugate (2)



Titration Curve

FIGURE 2

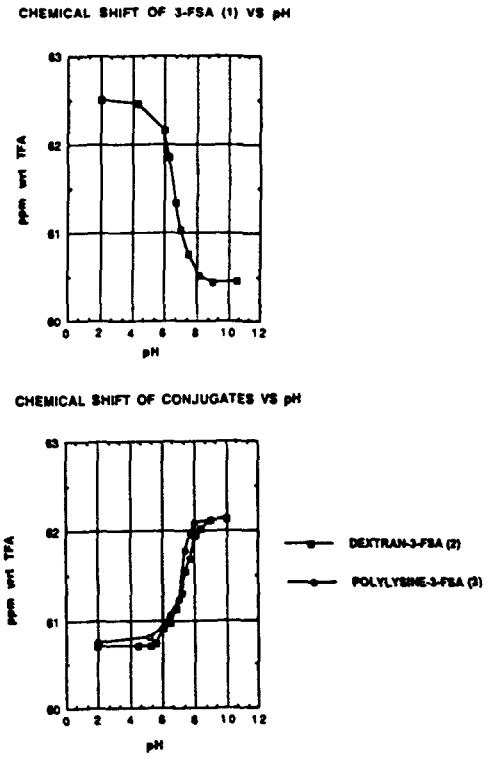


Table I: Characteristics of 3-FSA and its Conjugates

PRODUCT	pH	^{19}F NMR δ ppm[a]	pH SENSITIVITY δ ppm	YIELD[b]
1	2.00 10.00	-62.50 -60.45	2.05	—
2	2.00 10.00	-60.71 -62.12	1.41	76%
3	2.00 10.00	-60.75 -62.14	1.39	84%
4	2.00 10.00	-60.74 -62.10	1.36	80%
5	2.00 10.00	-61.00 -62 20	1.20	75%

a Chemical shift w.r.t. TFA, b Based on amount (mg) of polymer recovered,

The Polymer-3-FSA conjugates (2 to 4) exhibit a single sharp ^{19}F NMR signal and have moderate ^{19}F pH sensitivity and the physiological pKa necessary for non-invasive *in vivo* measurement of pH by ^{19}F MRS. We are developing both enhanced MR techniques and fluorinated functional NMR imaging agents to determine diverse physiological parameters. To the best of our knowledge, this is the first report describing the conjugation of a ^{19}F pH marker to a carrier molecule to create functionalized polymeric probes for non-invasive *in vivo* assessment of pH by ^{19}F MRI/MRS. Further studies are underway to synthesize macromolecular pH probes with enhanced pH sensitivity and demonstrate their *in vivo* utility.

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19. Coupling of 3-FSA(1) to polymers: 3-FSA was added to 0.5% K_2CO_3 solution of the polymer. It was allowed to stir overnight followed by addition of $NaBH_4$ and stirring at 37 °C for 2 h. The mixture was exhaustively dialyzed against water and lyophilization yielded Polymer-3-FSA conjugate (**2-4**).
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22. Coupling of 3-FSA(1) to aniline: 3-FSA and aniline were dissolved in methanol. The mixture was refluxed for 6 h. $NaBH_4$ was added to the mixture and stirred for 2 h. The product **5**, was obtained by column chromatography (hexane/choloroform; 8 : 2) followed by crystallization with hexane. mp 114-115 °C; MS (m/z, FAB): 218 $(M+1)^+$.
23. 2M phosphate buffer solution was used for albumin and 5% ammonium dihydrogenphosphate adjusted to pH -4.0 (using phosphoric acid)+ 3% ACN for polylysine.
24. Precise pKa of products 4 and 5 could not be determined, because these materials came out of solution at neutral pH.