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New flourinated chitin derivatives: synthesis, characterization and cytotoxicity assessment

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Abstract

New fluorinated chitin derivatives have been synthesized and characterized. Fluorination of chitin was achieved by facile homogenous reaction of chitin solution with diethyl amino sulfur trifluoride ($C_4H_{10}NSF_3$). The degree of substitution of the C6-hydroxyl functionality of *N*-acetyl-glucosamine repeat unit ranged from 50 to 98%, achieved by varying the reaction time from 1 to 144 h at room temperature. The use of pentafluoropropionic anhydride, trifluoromethylbenzoyl chloride and pentafluorobenzoyl chloride gave fluoro-chitin derivatives with 40, 10 and 5% substitution, respectively. Solid-state nuclear magnetic resonance and Fourier-transform infrared spectroscopy, powder X-ray diffraction, and elemental analysis support the identity of all fluorinated chitin derivatives. The fluorinated chitin derivatives were subjected to MTT assay using human (ATCC CCL-186) and mouse (ATCC CCL-1) fibroblast cell lines. Fluorinated chitin derivatives prepared from $C_4H_{10}NSF_3$ at 1, 6, 12, 72 and 96 h showed good cell viability of 80–100% for human fibroblast and 60–70% for mouse fibroblast. The % cell viability for the other fluorinated chitin derivatives were above 60% for both cell lines.

Keywords: Fluorine containing chitin derivatives; MTT assay; Chitin derivatives; Chitin substitution

1. Introduction

The general interest in fluorinated polymers have been centered mainly on their unique stability, low dielectric constant, low surface energy and low water uptake (Gangal, 1989; Maruyama Oishi, Kakimoto & Imai, 1988; Nagata, Tsutsumi & Kiyotsukuri, 1988). The fluorine atom has a relatively smaller atomic size and higher electronegativity. Therefore, the C–F bond of deoxyfluorinated sugars can participate only as its proton acceptor in hydrogen bonding between C–F and hydroxyl or amino groups. This unique hydrogen bonding capability of C–F bonds, coupled with the affects on the acidity of neighboring protons, may induce various activities via specific interactions with the biological systems (Laurence, Mulard, Cornelis & Glaudemans, 1998; Jen & David, 1998).

To date, only examples of deoxyfluorocellulose have been reported (Kasuya, Iiyama & Ishizu, 1992; Kasuya, Iiyama, Meshitsuka & Ishizu, 1994; Kasuya, Iiyama, Meshitsuka & Okana, 1997). No other new derivatives were reported thereafter. Our research group has investigated the fluorination of chitin, a β(1 → 4)-linked 2-deoxy-2-acetamido-D-gluco-pyranosyl polysaccharide. Chitin is found widely in nature including arthropod shells and fungal cell walls. The composition of chitin is similar to cellulose except for the acetylated C-2 hydroxyl groups. Chitin, the second most abundant polysaccharide after cellulose, is susceptible to biodegradation in nature by enzymes such as lysozyme and chitinase (Kurita, 1997). The bromination, chlorination and iodination of chitin have been reported by various groups (Sakamoto & Tseng, 1994; Tseng, Furuhata & Sakamoto, 1995; Tseng, Tekechi & Furuhata, 1997). Fluorinated chitin derivatives have not been reported. In this paper, we present the first preparation, characterization and in vitro cytotoxicity assessment of deoxy-fluorinated chitin.

2. Experimental

Chitin was obtained from Polyscience, USA, and purified by stirring in 1 M NaOH at room temperature for seven days and in 1 M HCl for 1 h. The degree of acetylation was determined by Fourier transform infrared spectroscopy (FT-IR) (Baxter, Taylor and Roberts, 1992) and microanalysis to be ~80%. All other reagents were of analytical grade unless otherwise stated.

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2.1. Preparation of chitin solution

Anhydrous LiCl (5.0 g) was dried at 130°C for about 1/2 h, cooled inside a desiccator and dissolved in 100 ml of DMAc with magnetic stirring. Chitin flakes (0.5 g) was suspended in this solution and shaken overnight at 150 rpm, 4°C in a refrigerated shaking incubator to give 100 ml of 0.5% (w/w) of chitin solution in 5% LiCl/DMAc. The viscous clear solution was filtered through glass wool and stored in glass containers at room temperature.

2.2. Synthesis of deoxyfluorochitin (*1*)

200 ml of the 0.5% chitin solution was poured into a plastic container. Diethylamino sulfurtrifluoride ($C_4H_{10}NSF_3$) (2.5 ml) was added drop-wise into the stirring solution. The mixture was allowed to stir for 1 h at room temperature. The resultant solution was poured into 200 ml of deionized water. The resultant black precipitate was filtered, washed repeatedly with excess deionized water, filtered and vacuum dried to give (**1**) in 95% (w/w) yield. The experiment was repeated for stirring times of 6, 12, 24, 72, 96, 120 and 144 h.

2.3. Synthesis of 6-O-6-(pentafluoropropanoate) chitin (**2**), 6-O-6-(4-(trifluoromethyl) benzoate) chitin (**3**) and 6-O-6-(pentafluorobenzoate) chitin (**4**)

200 ml of the 0.5% chitin solution was heated to 80°C. 4.0 ml of pentafluoropriopionoic anhydride was added drop-wise into the stirring solution. The mixture was allowed to stir for another 48 h at 80°C. The resultant solution was poured into 200 ml of methanol. The brownish precipitate that formed in the methanol was filtered, washed repeatedly with excess methanol, filtered and vacuum dried to give (**2**) in 95% (w/w) yield. The experiment was repeated using 2.5 ml of 4-trifluoromethylbenzoylchloride to give (**3**) in 98% (w/w) yield and with 2.0 ml of pentaflurorobenzoylchloride to give (**4**) in 98% (w/w) yield.

2.4. Characterization

Elemental analyses were performed by the Elemental Analysis Laboratory of the Chemistry Department, National University of Singapore using a Perkin–Elmer PE 2400 CHN elemental analyzer for simultaneous determination of carbon, nitrogen and hydrogen. The Schoniger combustion method was used to establish the fluorine content in the chitin derivatives and the degree of fluorination of each derivative estimated using the F/N ratio.

FT-IR spectra were recorded with a Bruker IFS 28 FT-IR spectrometer. All samples were vacuum dried overnight, powdered and made into KBr discs for spectra acquisition. ^{13}C CPMAS (solid-state cross polarization magic angle spinning nuclear magnetic resonance (NMR) spectroscopy) spectra were obtained at room temperature with a Bruker Varian 400 MHz NMR spectrometer using a contact time of 2 ms and a rotor frequency set at 10 kHz. Proton cross-polarization and decoupling frequency were adjusted to the pure water resonance of 4.65 ppm. The ^{13}C transmitter frequency was adjusted to 100 ppm using adamantane as reference at 38.5 ppm for CH_2 peak, downfield. 1500 scans were acquired for each sample.

Differential scanning calorimetry (DSC) measurements were obtained with a TA Instruments 2920 scanning calorimeter (TA Instruments, USA) at a heating rate of 5°C/min from room temperature to 300°C. X-Ray diffraction patterns were measured with a Siemens D5005 X-ray Diffractometer set at 20 kV, 5 mA, $2\theta = 3–35°$ step size = 0.02° and time/step = 2 s. A Kristalloflex 760 X-ray generator with copper tube and nickel K filter was used as the laser source.

2.5. Cytotoxicity assessment

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was employed as a colorimetric cytotoxicity assay to estimate the number of viable cells after contact with the chitin derivatives (Mosmann, 1983; Heeg, Reimann, Kabelitz, Hardt & Wagner, 1985; Green, Reade & Ware, 1984; Denizot & Lang, 1986). Reduction of the tetrazolium salt into a blue colored product (formazan) occurs only in metabolically active cells. The amount of formazan produced is proportional to the number of living cells. By measuring the UV absorbance of the formazan at 570 nm with a reference wavelength at 650 nm, the percentage of viable cells was estimated. The cell lines used in the MTT assays were NCTC Clone 929 (ATCC CCL-1) mouse fibroblast and IMR-90 (ATCC CCL-186) human fibroblast from American type culture collection (ATCC). Both lines were grown and maintained in Eagle's minimum essential medium with 1.5 g/l sodium bicarbonate and Earle's BSS adjusted to contain 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum, at 37°C in 5% CO_2 atmosphere. All medium supplements were from Gibco.

Cell suspensions (1×10^4) were inoculated into 96-well microtiter plates in 100 μl EMEM medium with 10% fetal bovine serum. Samples were added to each well (eight replicates per sample) and incubated at 37°C in a humidified atmosphere and 5% CO_2 for 24, 48, 72 and 96 h. After the specified exposure time, 20 μl of MTT solution was added into each well and incubated for another 3 h at 37°C. The medium was removed from the wells and the cells were washed very carefully with phosphate buffered saline (PBS — pH 7.5) to remove unreacted MTT and sample residues. Subsequently, 150 μl of dimethyl sulfoxide (DMSO) was added to each well to completely solubilize the blue formazan crystals. The absorbance of the DMSO solution was measured using a microplate spectrophotometer CERES UV 900C, Bio-Tek Instruments, at 540 nm. Results of the assays are reported as percentage

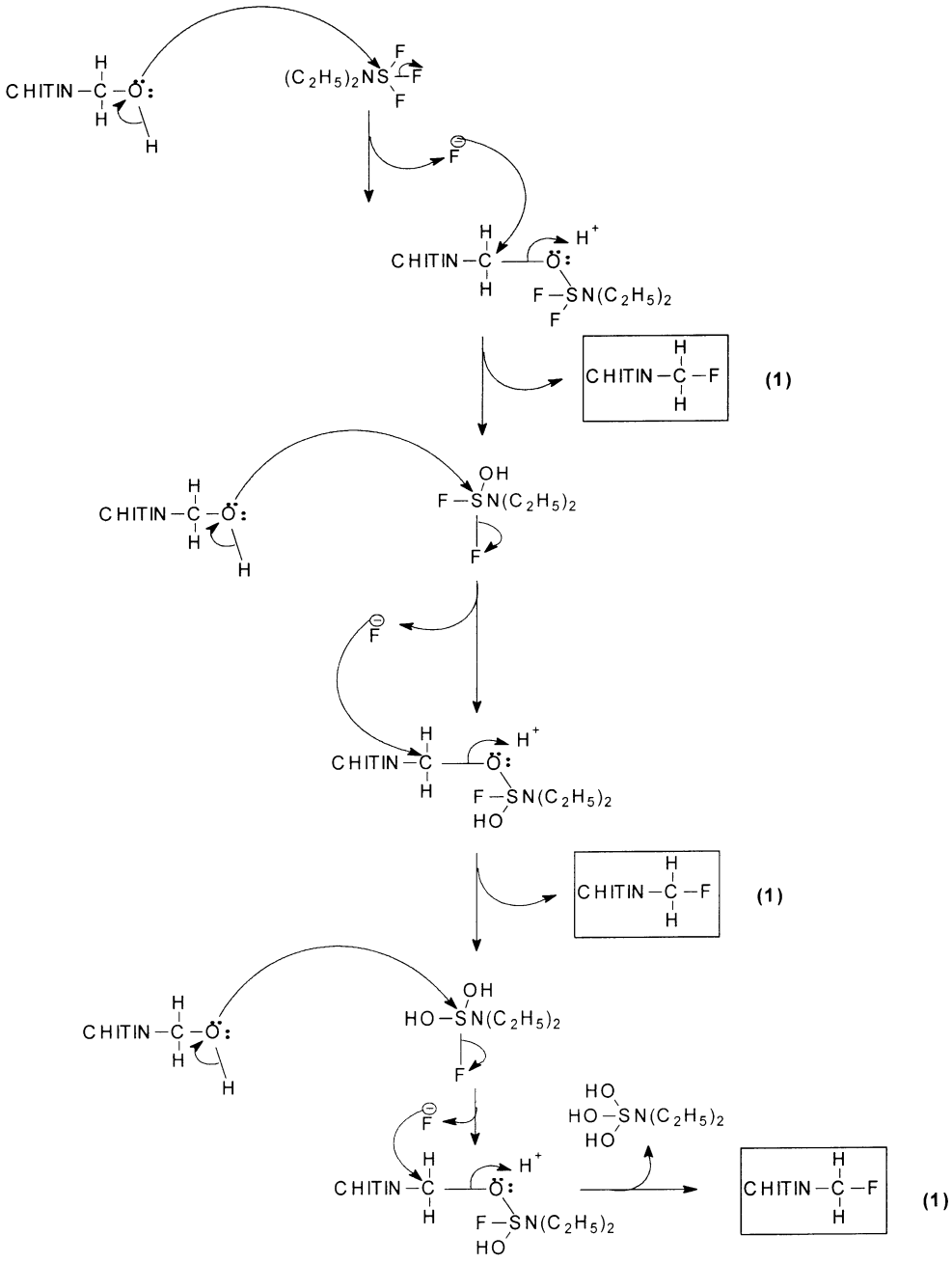

Fig. 1. $C_4H_{10}NSF_3$ mechanism for fluorination of chitin (**1**).

of absorbance relative to a control without exposure to any samples.

3. Results and discussion

3.1. Preparation of fluorine derivatives of chitin

The fluorination of chitin with diethylamino sulfur trifluoride ($C_4H_{10}NSF_3$) was adapted from the reaction between $C_4H_{10}NSF_3$ and cellulose 2,3-diacetate and cellulose 2,3-dibenzoate (Kasuya et al., 1994; Kasuya, et al., 1992; Kasuya, et al., 1997). Fig. 1 outlines the reaction pathway for the substitution of fluoride ion from $C_4H_{10}NSF_3$ by the electronegative hydroxyl group in chitin. Subsequent nucleophilic attack by free fluoride ion gives the deoxyfluoro chitin (**1**). The extent of this process is mediated by the exposure time to $C_4H_{10}NSF_3$. Therefore, for each mol equiv. of $C_4H_{10}NSF_3$, 3 mol equiv. of deoxyfluorochitin was produced. As the homogenous fluorination with $C_4H_{10}NSF_3$ preceded the viscosity of the system decreased. After stirring for about 20 h, the initial gel-like viscous property was no longer obvious but had transformed into a dilute fast flowing solution. No gelation was observed

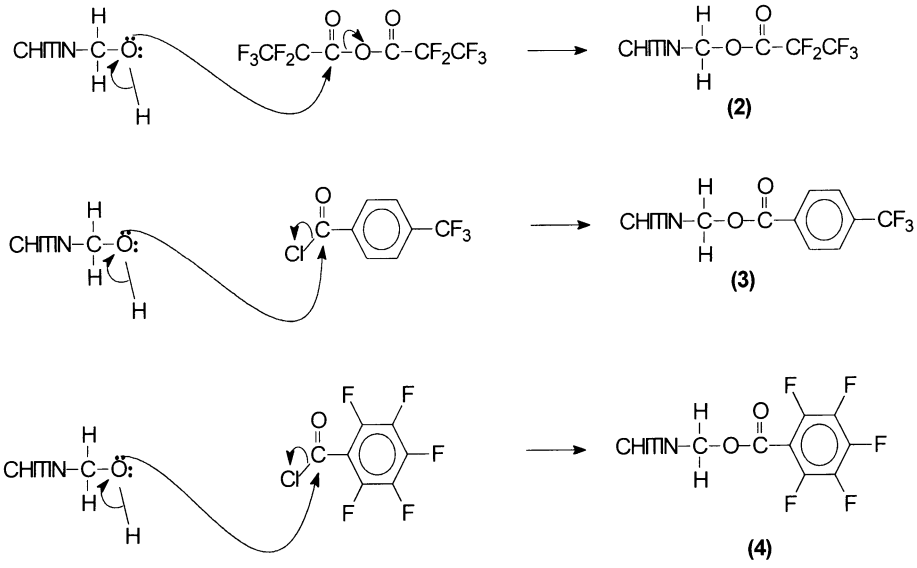

Fig. 2. Condensation reactions mechanism for fluorination of chitin (**2**), (**3**) and (**4**).

after addition of deionized water at the end of the reaction. The low viscosity indicates a low degree of inter/intra molecular hydrogen bonding interactions in the polymeric network. The addition of deionized water precipitated the product as a black powdery suspension. This appearance is very different from the yellowish white color of native chitin.

Most chitin reactions will produce an ether bond from a condenzation reaction between the C6-hydroxyl group and the substituting reagent (Kurita, Inoue & Nishimura, 1991; Miyazaki & Matsushima, 1968). Here, the reaction of chitin with $C_4H_{10}NSF_3$ results in a concurrent removal of the C6-hydroxyl group as well as, to a small extent, the C3-hydroxyl group as observed in previous halogenation reactions of chitin. The removal of oxygen at the C6 and C3-positions collapses the strong hydrogen bonding interactions between the polysaccharide chains resulting in the observations stated above. Other reported deoxychitin derivatives include the secondary reaction products of deoxybromochitin and deoxychlorochitin and 6-deoxychitin (Zhang, Inui & Hirano, 1997) and azidodeoxychitin (Furuhata, Arai, Ishizuka, Tseng & Sakamoto, 1998).

The reaction of chitin with pentafluoropropionic anhydride, 4-(trifluoromethyl) benzoyl chloride and pentafluorobenzoyl chloride gave the more traditional condensation products of chitin derivatization with the formation of an ester bond between the C6 hydroxyl group of chitin and the respective reagents (Fig. 2). The reaction between pentafluoropropionic anhydride and chitin solution gave the aliphatic fluorinated derivative of 6-*O*-6-(pentafluoropropanoate) chitin (**2**), whereas 4-(trifluoromethyl) benzoyl chloride and pentafluorobenzoyl chloride produced aromatic fluorinated derivatives, 6-*O*-6-(4-(trifluoromethyl) benzoate) chitin (**3**) and 6-*O*-6-(pentafluorobenzoate) chitin (**4**). All three mixtures remained homogenous throughout the period of reactions but were more viscous than $C_4H_{10}NSF_3$. This is attributed to the lower degree of substitution by these reactants that only partially eliminates the

Table 1
Elemental analysis results for fluorochitin (**1**), (**2**), (**3**), (**4**)

Compounds	Time (h)	% F	% N	Ratio F	Ratio N	Ratio of sub[a]	% Subs
(**1**)	1	2.96	4.38	0.16	0.31	0.31	50
	6	3.18	4.24	0.17	0.3	0.3	55
	12	3.59	4.57	0.19	0.33	0.33	58
	24	4.78	5.14	0.25	0.37	0.37	69
	72	3.07	3.02	0.16	0.22	0.22	75
	96	3.13	2.7	0.16	0.19	0.19	85
	120	3.67	2.88	0.19	0.21	0.21	94
	144	3.93	2.95	0.21	0.21	0.21	98
(**2**)		3.32	1.18	0.17	0.08	0.03	41
(**3**)		2.18	5.9	0.11	0.42	0.04	9
(**4**)		1.91	6.24	0.1	0.45	0.02	5

[a] Normalized for the number of fluorine atoms present.

Table 2
Cytotoxic assessment of fluorochitin derivatives (**1**), (**2**), (**3**) and (**4**)

	Compounds	Reaction time (h)	Cytotoxicity assay time (days)			
			1	2	3	4
Human fibroblast cell line (ATCC CLL-186)	(**1**)	1	92.97	97.08	83.47	96.38
		6	74.69	80.99	78.95	95.63
		12	87.48	87.71	83.30	102.56
		24	75.15	73.87	84.76	97.39
		72	85.10	73.79	92.59	86.92
		96	86.99	86.12	89.91	101.77
		120	75.15	74.49	77.8	76.09
		144	70.88	73.21	70.95	72.38
	(**2**)		85.9	98.4	91.34	93.22
	(**3**)		82.81	87.31	98.18	89.95
	(**4**)		90.22	88.5	95.66	94.21
Mouse fibroblast cell line (ATCC CLL-1)	(**1**)	1	64.63	74.94	100.4	103.25
		6	65.02	65.61	79.32	94.11
		12	73.79	68.05	90.7	101.6
		24	63.4	73.73	88.26	94.7
		72	62.52	65.3	81.25	100.92
		96	69.1	79.36	93.02	88.12
		120	48.19	49.87	49.62	44.91
		144	47.72	44.19	48.16	40.02
	(**2**)		85.13	56.51	64.6	76.15
	(**3**)		89.38	78.85	95.99	75.69
	(**4**)		67.21	66.57	62.04	69.58

strong hydrogen bonding interactions between the polysaccharide chains which renders the final products insoluble in common solvents.

It should be noted that all the mixtures remained homogenous throughout the reaction and only became insoluble after precipitation. This suggests that these chitin derivatives could be fabricated into desired shapes and forms immediately after the reaction that on precipitation retains the integrity of that shape or form. This permits a method of processing these chitin derivatives into useful end products.

3.2. Characterization

Table 1 summarizes the extent of fluorination achieved by all the fluorine reagents. Fluorine substitution with $C_4H_{10}NSF_3$ was 50% after 1 h and reached a saturation point of 98% substitution at about 144 h. In contrast, the condenzation reactions between chitin and pentafluoropropionic anhydride, 4-(trifluoromethyl) benzoyl chloride and pentafluorobenzoyl chloride, respectively, resulted in significantly lower substitutions. The difference in reactivity between $C_4H_{10}NSF_3$ and the other three fluorine reagents may be attributed to the reactivity and bulkiness of the reagents. $C_4H_{10}NSF_3$ is the smallest and most reactive, followed by pentafluoropropionic anhydride (40% substitution). Both 4-(trifluoromethyl) benzoyl chloride and pentafluorobenzoyl chloride have relatively bulkier structures and the degree of substitution rate were $<10\%$.

From DSC experiments, no derivatives showed any glass transition or melting temperatures. The fluorinated chitin derivatives decomposed from about 190–220°C. The lack of a melting temperature is disappointing and is attributed to residual hydrogen-bonding interactions that are still strong enough to prevent any flow or melt of the polymeric chains.

Chitin has 2θ diffraction peaks at 9.3, 19.4 and 26.3°, assigned to the corresponding diffraction planes of (020), (110) and (101) in the α-form orthorhombic crystalline structure (Zhang, Haga, Sekiguchi & Hirano, 2000). All the fluorinated chitin derivatives display reduced 2θ peaks intensity (exemplified by (**1**) with a reaction time of 144 h. The lower peak intensities were directly related to the reduction in molecular crystallinity of the derivatives. The introduction of reactant molecules into the polymeric chains does disrupt the strong inter/intra molecular network of hydrogen bonding giving a more amorphous material.

Solid-state ^{13}C NMR spectroscopy has proven useful in analyzing insoluble complexes of biopolymers. The primary peaks found with chitin ^{13}C CPMAS are the acetyl carbon at 23.47 ppm, C2 at 55.86 ppm, C6 at 61.50 ppm, C3 and C5 at 75.04 ppm, C4 at 84.00 ppm, C1 at 104.32 ppm and the carbonyl carbon at 174.37 ppm, similar to those reported in the characterization of exoskeleton chitin by Martin, Lothar & Hans, 1984. These chitin peaks are found in the

fluorinated derivatives, indicating the structural integrity of chitin. The NMR spectrum of (**1**) shows a broad spectrum in the region of ca. 60–80 ppm. The presence of fluorine broadens the sharp peaks originally present at these positions, similar to the H_2C–F and HC–F positions found in fluorinated cellulose (Kasuya et al., 1994). The ^{13}C CPMAS spectrum of (**2**), (**3**) and (**4**) showed broad peaks at ca. 100–140 ppm. Peaks in the region corresponded to the aromatic F and CF_3 as well as aliphatic CF_2CF_3, reported in the ^{13}C NMR spectrum of fluorobenzene, 2-trifluoromethyltoluene and perfluoro-*n*-octylethyl alcohol, respectively (Oishi, Kawamoto & Fujimoto, 1994; Freund & Jung, 1989). The carbonyl peaks present in these three derivatives were not visible in the spectra due to masking by the carbonyl peak of acetyl moiety from chitin.

The FT-IR spectra of the fluorinated chitin derivatives shows very little differences between the derivatives and chitin, with characteristic absorptions appearing at ca. 1655 cm^{-1} for the carbonyl stretching vibration (amide I) and at ca. 1560 cm^{-1} corresponding to the NH deformational vibration (amide II). Only an enhancement between 500–700 cm^{-1} was observed. These peaks are C–F bonds that vibrate at the lower end of the IR region. The presence of the ester linkage between the fluorine containing substituents and chitin in derivatives (**2**), (**3**) and (**4**) is indicated at ca. 1760 cm^{-1}. The ester peaks found in the FT-IR spectra is indicative of substituents that are covalently bonded to chitin by reaction between the C6 hydroxyl group and the fluorinated reagents.

3.3. Cytotoxicity assessment

Results for cytotoxicity assessment over a period of four days of the fluorinated chitin derivatives were tabulated in Table 2. The deoxyfluoro-chitin derivatives (**1**) prepared by reaction with $C_4H_{10}NSF_3$ at 1, 6, 12, 24, 72 and 96 h gave cell viability values of between 80–100% in human fibroblast cultures. The derivatives obtained at 120 and 144 h gave a lower% cell viability of 60–70%. A similar trend was found for the mouse fibroblast cell line, with the derivatives obtained at 120 and 144 h showing lower cell viability at 40–50%. The lower cell viability for the higher fluorine content derivatives suggest that there is an optimum level for fluorine incorporation above which the fluorine content exerts an undesirable effect. This conclusion is further supported with the other fluorine containing chitin derivatives (**2**), (**3**) and (**4**), where the lower degree of fluorination is translated as higher cell viability (>60%). Another observation is that mouse fibroblast cells lines give a lower% cell viability (up to a 20% difference) compared to human cells. This observation is intriguing and warrants further investigation.

4. Conclusion

The preparation of fluorine chitin derivatives has been achieved. Controlled fluorination with $C_4H_{10}NSF_3$ is possible giving a range of degree of substitution between 50–98% depending on reaction time. Fluorination via condenzation reaction is dependent on the structural bulkiness and reactivity of the reactant, with the pentafluoropropionic anhydride producing a higher substitution at 40% compared to 4-(trifluoromethyl) benzoyl chloride and pentafluorobenzoyl chloride at <10%. MTT assay shows that fluorinated chitin materials are in general, non-cytotoxic and this property appears to be a function of the degree of fluorine present in the derivative. This work offers the prospect of fabricating fluorinated chitin derivatives as potential materials for various applications including biomedical.

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