Direct qualitative and quantitative characterization of a radiosensitizer, 5-iodo-2′-deoxyuridine within biodegradable polymeric microspheres by FT-Raman spectroscopy

The Analyst
Perspective

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Non-destructive qualitative and quantitative characterization of a radiosensitizer, 5-iodo-2′-deoxyuridine (IdUrd), incorporated within injectable microspheres of a biodegradable polymer, poly(D,L-lactide-co-glycolide) (PLGA), was performed using Fourier transform (FT) Raman spectroscopy. Raman spectra of IdUrd, free and entrapped in microspheres, were recorded under fluorescence-free conditions, described and assigned. For the Raman bands of the PLGA microspheres, assignments with preferential localization of the corresponding vibrations at lactic or glycolic units were proposed. No evidence for drug–polymer interactions in microspheres was found. This allowed the FT-Raman spectra to be used for the quantification of the IdUrd content in the samples. For the microspheres with IdUrd loadings varying from 2 to 27% of the total weight, the methodology used provided good reproducibility and precision (1%). Within the sensitivity of the technique, samples exposed to sterilization doses (27 kGy) of γ-radiation did not exhibit marked changes in the drug structure.

Nowadays, Raman spectroscopy has become an easily used technique with a very wide range of applications. For the resolution of numerous analytical problems, the molecular-specific information obtained in a non-destructive way seems to be irreplaceable. Moreover, Raman spectroscopic data can be successfully used for quantitative measurements. Owing to some particular advantages, such as fluorescence-free conditions, Fourier transform (FT) Raman spectroscopy with excitation in the infrared region is especially useful in analytical studies of raw samples.

In this study, we aimed to apply FT-Raman spectroscopy to the non-destructive qualitative and quantitative characterization of a drug incorporated within a polymeric matrix. The polymers concerned are bioresorbable aliphatic polyesters based on copolymers of lactic (LA) and glycolic acid (GA). These biocompatible polymers degrade hydrolytically in the body with the formation of non-toxic products.[1] Poly(D,L-lactide-co-glycolide) (PLGA) polymers have been studied as materials for osteosynthesis, sutures and prosthetic devices.[1] They are widely used for therapeutic purposes especially to make sustained drug release delivery systems.[2]

We focused on PLGA-based delivery systems as used for the sustained release of 5-iodo-2′-deoxyuridine (IdUrd). This molecule is a thymidine analog and is a powerful radiosensitizer.[3] This halogenated pyrimidine competes with thymidine in the biosynthesis of DNA. The treatment of malignant brain tumors based on conventional radiotherapy is far from satisfactory. Therapeutic IdUrd concentrations within the tumor during the time course of radiotherapy might improve treatment results, by increasing the lethal effects of radiation on the tumor cells having incorporated the radiosensitizer. The intracranial implantation of IdUrd-loaded microparticles in the vicinity of the cancer cells can meet these requirements.[4]

The study involved the preparation of PLGA microspheres loaded with different amounts of IdUrd, by using a phase separation technique. Intact IdUrd-loaded microspheres were characterized qualitatively and quantitatively by FT-Raman spectroscopy. Since the microspheres are intended to be administered *in vivo* into the brain, they need to be sterile. The sterilization of biodegradable drug delivery systems is often carried out by γ-irradiation. The stability of the drug entrapped in the microspheres[5,6] after exposure to γ-radiation was determined from the respective Raman spectra.

Experimental

Chemicals

IdUrd (99% pure, odorless, white, crystalline powder, slightly soluble in water: 2 mg ml^{-1}) was obtained from Sigma-Aldrich Chimie (St. Quentin Fallavier, France). PLGA 50/50 was purchased from Boehringer Ingelheim (RG 506, B.I. Chimie, Paris, France). The composition of the chains includes 25% L-lactic units, 25% D-lactic units and 50% glycolic units. The mass- and number-average molecular masses were 75 000 and 48 000, respectively. These values were determined in tetrahydrofuran by size exclusion chromatography (SEC Waters, St. Quentin en Yvelines, France), referred to polystyrene standards. Methylene chloride, silicone oil (Rhodorsil, viscosity 300 cSt, relative density 0.97) was obtained from Prolabo (Paris, France). Heptane was purchased from Verbièse (Wasquehal, France) and dimethyl sulfoxide (DMSO) from Carlo Erba (Val de Reuil, France).

IdUrd crystal milling

Grinding of IdUrd crystals was performed with a Pulverisette 7 planetary micro-mill (Fritsch, Idar-Oberstein, Germany). A 800 mg amount of IdUrd was milled for 10 min at a rotation speed of 2500 rpm.

Microsphere preparation

The coating polymer PLGA (250 mg) was dissolved in methylene chloride to reach a concentration of 1.3% m/m. Various amounts of milled IdUrd crystals (18 ± 3 μm, SD of different mean size values) were then dispersed in the organic phase with sonication for 3 min. A separation phase inducer (silicone oil, 8 g) was added to the mixture and stirred magnetically at room temperature for 2 min in order to precipitate the polymer around the drug particles. The resulting dispersion (semi-formed microparticles or coacervates) was poured into 400 ml of heptane (hardening agent), stirred at 600 rpm (Heidolph RGH500, Prolabo, Paris, France). After 30 min of agitation, the solidified microparticles were filtered on a 0.45 μm filter (HV type, Millipore, Maurepas, France) and washed with heptane (50 ml). The resulting microspheres were dried under reduced pressure for 60 h at 35 °C. They were stored at 6 °C shielded from light.

Microsphere size distribution analysis

The average size of the microparticles was determined using a Coulter Multisizer (Coultronics, Margency, France) after dispersion of the microparticles in a conducting liquid (Isoton II, Coultronics).

Crystal size distribution

The average size of the crystals was determined using a Mastersizer S (Malvern Instruments, Malvern, Orsay, France). Size measurements were performed in the liquid phase, using a 300 RF lens, in the size range 0.05–880 μm and an MSI module as a manual liquid sampler. A 30 mg amount of milled powder was suspended in 3 ml of cyclohexane with sonication and immediately analyzed.

γ-Irradiation of microspheres

IdUrd-loaded microspheres were accurately weighed into 100 mg samples, transferred to 2.5 ml glass vials and sealed. The vials were irradiated at a dose of 26.7 kGy ^{60}Co source, (Ionisos, Dagneux, France). This was done in triplicate.

IdUrd content determination

This was achieved using two methods, spectrophotometry and Raman spectroscopy. The former method provides direct access to the drug concentration *via* molar absorptivity (7.9×10^3 l mol^{-1} cm^{-1} at 287 nm) but was destructive, since the microspheres (6–8 mg) needed to be dissolved in DMSO. The latter method, allowing non-destructive quantification of IdUrd, was developed using the Raman spectra obtained from intact microspheres. These spectra contained a contribution of the polymer vibrations which could be useful as an internal standard. The IdUrd/PLGA peak area ratio of the Raman bands allowed the calculation of the corresponding ratio of the concentrations of these molecules. This is discussed below.

Spectroscopic instrumentation

The absorption of IdUrd was measured with a Uvikon 922 UV spectrophotometer (Kontron Instruments, St. Quentin en Yvelines, France). The IdUrd solutions were shielded from light. The Raman spectra were excited with 1.06 μm radiation from an ADLAS DPY 301 Nd:YAG laser and recorded with a Bruker (Wissembourg, France) RFS100/D418-S FT-Raman spectrometer. The laser power at the sample was about 140 mW. No laser-induced sample degradation was noted during the experiments. All the measurements were repeated at least three times. The data appeared to be well reproducible.

The data obtained were analyzed with Labspec software (DILOR, Lille, France), which permitted the very easy and rapid treatment of the spectra (baseline correction, peak area analysis, normalization, *etc*.) and statistical analysis of any spectral parameters. In addition, this software package allowed us to treat simultaneously and in exactly the same manner all the sets of the recorded spectra.

Results and discussion

In order to analyze the Raman spectra of the drug-containing microspheres, the model Raman spectra of the blank PLGA 50/50 microspheres and of IdUrd crystals (Fig. 2) were recorded.

FT-Raman spectra of IdUrd crystals

In the FT-Raman spectra of IdUrd crystals (Fig. 2 and Table 1), both the bands of iodo-substituted aromatic nucleus (5-iodouracil) and those of the deoxyribose moiety (Fig. 1) can be observed. As a result, the spectra appeared very rich in vibrations, especially within the 1400–1800 cm^{-1} region. Since the Raman spectra of IdUrd have not been reported previously, we describe them in detail. Our discussion is limited to only the more intense bands between 1800 and 700 cm^{-1}. The CH region bands above 2800 cm^{-1} and the lower wavenumber weak deformational bands were less interesting with respect to the main task of the present study.

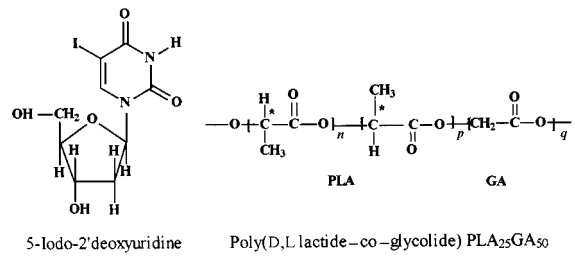

Fig. 1 Structural formulae of IdUrd and PLGA.

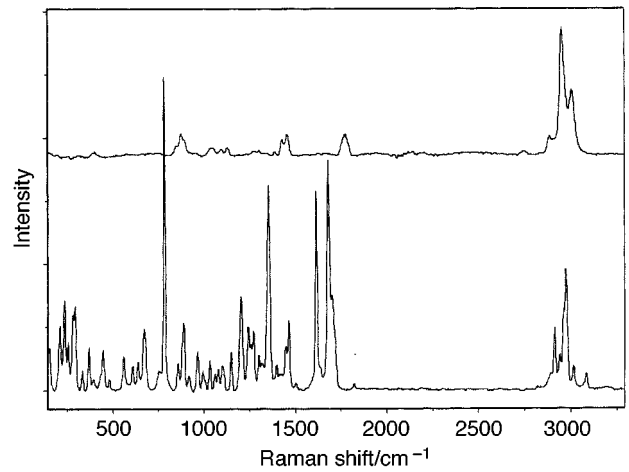

Fig. 2 FT-Raman spectra of IdUrd crystals (bottom) and the blank PLGA microspheres (top).

As expected for the IdUrd molecule, the very characteristic stretching bands of the non-conjugated and conjugated C = O groups were clearly observed at 1696 and 1676 cm^{-1}, respectively (Fig. 2 and Table 1). In their neighborhood, at 1611 cm^{-1}, one could observe another strong band, that of the ν(C = C) vibration. The region of the CH_2 deformation motions was represented by a weak doublet at 1460/1444 cm^{-1} encircled with two even weaker bands which are not discussed here (see Table 1). A strong band at 1350 cm^{-1} had the characteristic wavenumber of H–N–C = O stretching (amide III). A group of weak bands located at lower wavenumbers, down to 1230 cm^{-1}, was due to CH deformation and CH_2 twisting. The bands within the 1200–1140 and 1105–1020 cm^{-1} regions were assigned to asymmetric and symmetric COC stretching, respectively. The vibrations observed between 1010 and 850 cm^{-1} were attributed to the C–C stretching and CH_2 rocking. The very strong band at 779 cm^{-1} was assigned to a ring breathing mode of the 5-iodouracil moiety. The Raman bands of IdUrd located near 750 cm^{-1} (δC = O) and lower were those of the various deformational motions of the C = O, CCO, *etc*., groups.

The FT-Raman spectra of IdUrd after mechanical milling (see Experimental) were devoid of any detectable changes (data not shown). Based on this and on the data from X-ray diffraction (not shown), it was concluded that no polymorphic forms were present in detectable amounts in these samples. Therefore, the crystallinity of IdUrd was preserved after milling.

FT-Raman spectra of the blank PLGA 50/50 microspheres

FT-Raman spectra of blank microspheres (Fig. 2 and Table 2) were analyzed in comparison with previously reported Raman spectra (in the visible region) of poly(D,L-lactide) (PLA)[7] and poly(glycolide) (PGA)[8] polymers.

The band positions and shapes in the spectra of PLGA microspheres indicated an amorphous form of polymer.[9,10] In general, the PLGA spectra contained both the PLA and PGA Raman bands, the former being more pronounced (see Table 2). With respect to this comparison, we proposed a temptative attribution of the observed PLGA bands to vibrational modes of lactic (LA) or/and glycolic (GA) units (Table 2).

Whereas both PLA and PGA spectra have already been well documented,[7-10] to our knowledge, there has been no report on the Raman spectra of PLGA. For this reason, only some particular features in the Raman patterns which differentiate the PLGA samples from PLA and PGA (Table 2), are discussed.

Table 1 Major Raman wavenumers (cm^{-1}) and their tentative assignments for IdUrd in pure crystals and when in PLGA microspheres[a]

Microspheres with IdUrd	Blank microspheres	Assignment	Crystals of IdUrd	Assignment
3080 w			3080 m	$\nu_{as}CH$
3010 w			3010 m	$\nu_{s}CH$
3002	3002 s	$\nu_{as}CH_3$		
2965 sh			2968 ν_s	$\nu_{as}CH_2$
2953 s			2956 s	$\nu_{as}CH_2$
2947 vs	2947 vs	$\nu_sCH_3 + \nu_{as}CH_2$		
2936 m			2936 m	$\nu_{as}CH_2$
2908 sh			2908 m	νCH
2876 s	2876 s	νCH		
1769 s	1769 s	νC = O		
~1760 sh	~1760 sh	νC = O		
1696 m			1696 m	νC = O, non conj.
1676 s			1676 s	νC = O, conj.
1611 s			1611 s	νC = C
1458 s	1452 s	$\delta_{as}CH_3 + \delta CH_2$	1460 mw	δCH_2
1442 sh			1445 w	δCH_2
1427 m	1427 m	δCH_2		
1390 w	1384 w	δ_sCH_3	1395 w	δCH_2
1350 s	1345 vw	$\delta_1CH + \delta_sCH_3$	1350 s	Amide III + δCH
1300 w	1303 w	δ_2CH	1296 w	δCH
	1274 vw	$twCH_2$		
1268 w			1267 mw	$twCH_2$
			1252 vw	$twCH_2$
1239 w			1239 mw	$twCH_2$
1199 mw			1198 m	ν_{as}COC
1145 w			1146 w	ν_{as}COC
1130 m	1130 m	$r_{as}CH_3$		
			1102 vw	ν_sCOC
1093 w	1093 w	ν_sCOC	1096 w	ν_sCOC
			1075 vw	ν_sCOC
			1055 vw	ν_sCOC
	~1048 sh	νC–CH_3		
1032 w	1032 w	ν_sCOC	1028 w	ν_sCOC
990 w			989 w	νC–C + rCH_2
961 w			961 w	νC–C + rCH_2
	953 vw	νC–C + rCH_2 + rCH_3		
	920 vw	νC–C + rCH_2	913 w	νC–C + rCH_2
	892 m	νC–C + rCH_2		
885 m			886 m	νC–C + rCH_2
874 s	874 s	νC–COO	878 m	νC–C + rCH_2
849 sh	847 mw	rCH_2	855 mw	rCH_2
779 vs			779 vs	Ring breathing
746 w	750 vw	δC = O	756, 749 w	δC = O
706 vw	706 vw	γC = O + δC = O		

[a] Abbreviations: v = very; s = strong; m = medium; w = weak; sh = shoulder; subscript s = symmetric; subscript as = asymmetric.

The wavenumbers that appeared significantly shifted in PLGA are given in italics in Table 2. This particularly concerned the stretching (3002 and 2947 cm^{-1}) modes of the CH_3 (LA) and CH_2 (GA) groups (Table 2). The interesting observation was that the copolymer formation seemed to affect the δCH_2 (1427 cm^{-1}) more than the δCH_3 (1384 cm^{-1}) wavenumbers and the $\delta_1 CH$ (1345 cm^{-1}) more than the $\delta_2 CH$ (1303 cm^{-1}) wavenumbers. The perturbations of the $\nu C–C$ and rCH_2 modes (both 920 and 892 cm^{-1}) were less predictable than those for $\delta C=O$ (750 and 706 cm^{-1}).

FT-Raman spectra of the PLGA microspheres loaded with IdUrd

These logically contained both drug and polymer bands (Fig. 3 and Table 1). We subtracted from these spectra the model spectra of the pure drug and blank microspheres (Fig. 4). The difference spectra, even obtained from those with a significant contribution of the polymer or drug to the Raman bands, did not reveal any noticeable changes as compared with their respective model Raman patterns. Therefore, no indication either of structural modification of IdUrd and/or PLGA matrix or of their interactions could be discerned. It was also noted that unchanged spectra (in wavenumbers and relative intensities) could be considered as the sum of the model spectra. This allowed the quantitative considerations discussed in the following section.

Determination of Raman IdUrd content in the IdUrd-loaded PLGA microspheres

The methodology used with incorporation of a phase separation technique yielded PLGA microspheres of nearly regular size in the range 40 ± 5 μm (SD on mean size of different microsphere batches) as established by particle sizing.

The first step in the current work was to establish the calibration curve, *i.e.* the function describing the Raman spectral parameters as a function of the drug/polymer relative concentrations. For this purpose, microspheres with different IdUrd loadings, ranging from 2 to 27% [IdUrd (mg)/microspheres (mg) × 100: incorporation ratio, determined using spectrophotometry] were analysed.

Comparison of the Raman spectra of the drug-loaded microspheres with the spectra of the pure IdUrd crystals and blank PLGA microspheres (Fig. 3) revealed several regions devoid of superposed bands of these molecules. In particular, in the region between 1600 and 1820 cm^{-1}, the drug and polymer vibrations were in a close neighborhood but well separated. This made these vibrations usable as an internal standard. Three strong Raman bands of IdUrd, at 1696, 1676 and 1611 cm^{-1}, and a very large band of PLGA with a maximum at about

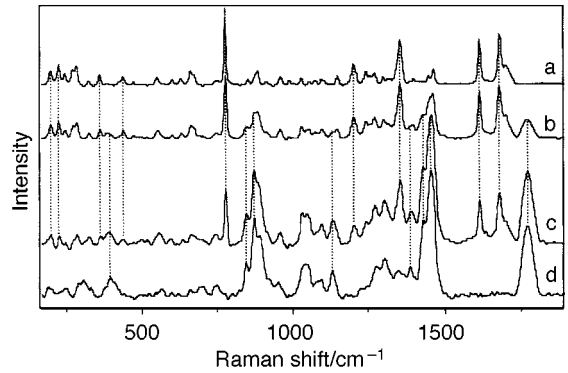

Fig. 3 Low-wavenumber region of the FT-Raman spectra: (a) IdUrd crystals; (d) blank PLGA microspheres; (b) and (c) PLGA microspheres containing different amounts of the drug. To emphasize the comparison, the spectra have been normalized using $\nu C=O$ bands (see Table 1) of the drug [(a) and (b)] or of the polymer [(c) and (d)].

Table 2 Major Raman wavenumbers (cm^{-1}) and their tentative assignments for PLGA 50/50 microspheres compared with PLA and PGA polymers[a]

Raman (λ_{ex} 514.5 nm)[b,c]		FT-Raman (λ_{ex} 1064 nm):		
PLA amorphous[b]	PGA amorphous[c]	PLGA50/50 microspheres	Localization[d]	Assignment
2997 s		*3002 s*	LA	$\nu_{as}CH_3$
2942 vs		*2947 vs*	LA +	$\nu_s CH_3$ +
	2954 vs		GA	$\nu_{as}CH_2$
2877 m		2876 s	LA	νCH
1769 s		1769 s	LA	$\nu C=O$
	1760 s	~1760 sh	GA	$\nu C=O$
1455 s		1452 s	LA +	$\delta_{as}CH_3$ +
	1450 sh		GA	δCH_2
	1423 s	*1427*	GA	δCH_2
	1400 sh	—		wCH_2
1386 m		1384 w	LA	$\delta_s CH_3$
1365, 1355 m		*1345 w*	LA	$\delta_1 CH + \delta_s CH_3$
1296, 1300 s		1303 w	LA	$\delta_2 CH$
	1274 m	1274 w	GA	$twCH_2$
1128 s		1130 m	LA	$r_{as}CH_3$
1092 s	1090 w	1093 w	LA + GA	$\nu_s COC$
1042 s		~1048	LA	$\nu C–CH_3$
	1029 m	1032	GA	$\nu_s COC$
953 sh	950 m	953,	LA +	$rCH_3 + \nu C–C +$
			GA	$\nu C–C + rCH_2$
		920		
	885 s	892 m	GA	$\nu C–C + rCH_2$
873 vs		874 s	LA	$\nu C–COO$
	845 s	847 w	GA	rCH_2
740 m		*750 vw*	LA	$\delta C=O$
700 vw		*706 vw*	LA +	$\gamma C=O$ +
	720 w		GA	$dC=O$

[a] Abbreviations: [b] As referred in ref. 7. [c] As referred in ref. 8. [d] With respect to the D, L lactic (LA) or glycolic (GA) units. v = very strong; s = strong; m = medium; w = weak; sh = shoulder; subscript s = symmetric; subscript as = asymmetric. Values in italics are wavenumbers shifted for PLGA compared with those of PLA and/or PGA.

1769 cm^{-1} were observed (for assignments, see Tables 2 and 3). Therefore, this spectral region was selected for quantitative measurements. Hereafter, the peak area ratio of the 1586–1723/1723–1815 cm^{-1} spectral regions, representing the drug/polymer Raman spectral ratio, R_d/R_p, was considered.

R_d/R_p plotted as a function of the respective incorporation ratio (%) is presented in Fig. 5. These experimental points fitted well a second-order polynomic curve (Table 3). In the range of incorporation ratios used for clinical applications (16–23%) the calibration curve was quasi-linear. This could be used to simplify the quantitative calculations for the commonly used incorporation ratios. The largest deviations of the observed R_d/R_p value from the fitted curve gave higher deviations of IdUrd incorporation within ±1% (see the shaded area in Fig. 5). Therefore, the reproducibility of the data was good and the approach allowed the determination of the IdUrd content in 'non-calibrated' microspheres from their Raman spectra with a precision of at least 1%.

Effects of exposure to γ-rays on IdUrd-loaded PLGA microspheres

Two sets of microspheres, loaded with 20 and 27% of IdUrd, were exposed to 27 kGy of ionizing radiation. A dose of 25 kGy was considered to be the minimum necessary for sterilization specified by the European Pharmacopoeia.

Strong ionizing radiation is known to be destructive for the polymeric matrix. Several reports have commented on γ-ray irradiation-induced chain scission and cross-linking[11-13] in polylactide and polyglycolide. However, concerning the active agent, the influence of irradiation on the drug structure remained to be defined. This point was essential since microspheres must be radiosterilized before implantation. In the present study, we focused on the irradiation effect on the sole IdUrd structure.

As followed from both spectrophotometric and Raman data, the irradiated PLGA microspheres exhibited nearly the same (±1%) IdUrd content as before the irradiation process. The FT-Raman bands of the irradiated IdUrd crystals, free or when incorporated within the microspheres, also remained unchanged, both in wavenumers and intensities (Fig. 6). This was also supported by the infrared absorption spectra (data not shown) of the same samples. Therefore, no drug degradation was found. This was a promising observation with respect to clinical application.

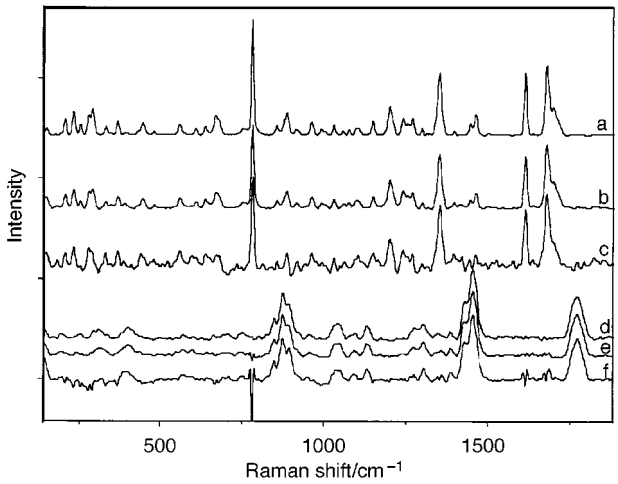

Fig. 4 (a) Model FT-Raman spectrum of IdUrd crystals; (b) and (c) difference spectra obtained by subtraction of the polymer model spectrum from those of the microspheres loaded with (b) 27% and (c) 4% of IdUrd; (d) model FT-Raman spectrum of the blank PLGA microspheres; (e) and (f) difference spectra obtained by subtraction of the drug model spectrum from those of the microspheres loaded with (e) 4% and (f) 27% of IdUrd. The difference spectra have been normalized for comparison with corresponding model spectra.

Table 3 Results of fitting of the R_d/R_p data to a second-order curve: $y = a + bx + cx^2$

Parameter	Value	Standard error	95% confidence interval
A (fixed constant)	0.0		
B	0.028141	0.003231	0.0202343–0.0360485
C	0.001818	0.000141	0.00147298–0.00216377
Degrees of freedom	6		
r_2	0.998181		
Absolute sum of squares	0.007489		
S_{yx}	0.0353		
No. of *x* values	10		
No. of *y* replicates (mean analyzed)	5		
Total no. of values	8		
No. of missing values	42		

Conclusion

We interpreted each of the FT-Raman spectra of IdUrd-loaded PLGA microspheres in both qualitative and quantitative ways.

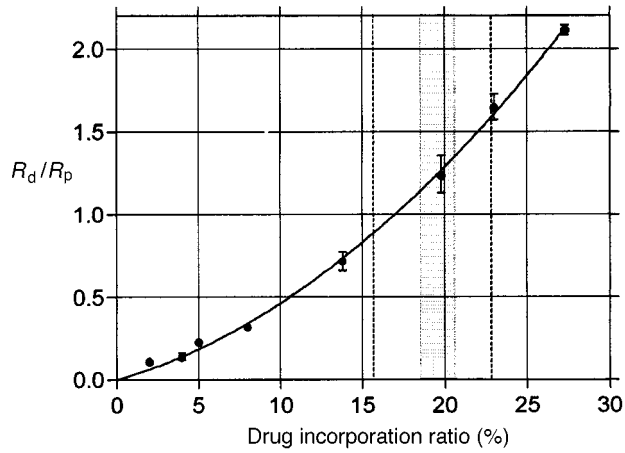

Fig. 5 Drug/polymer Raman peak area ratio, R_d/R_p, *versus* IdUrd incorporation ratio (%).

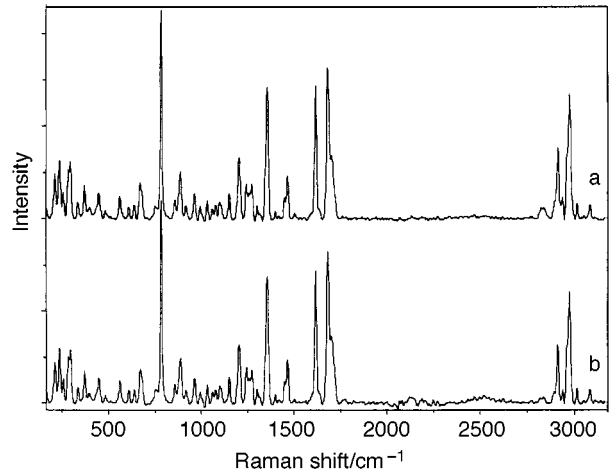

Fig. 6 Comparison of the FT-Raman pattern of IdUrd in PLGA microspheres (a) before and (b) after irradiation with 27 kGy of γ-rays. These are difference spectra obtained by subtraction of the model spectra of the blank microspheres from the spectra of the microspheres loaded with 20% of IdUrd.

The simultaneous access to both qualitative and quantitative information illustrated the major advantages of the proposed analytical approach. The qualitative information included the detailed analysis of the spectral shape, *i.e.*, band position and relative intensity. This allowed the molecular characterization of the samples and therefore detection of probable structural changes of the drug and/or polymer matrix induced by interaction, degradation, *etc*. The quantitative analysis included the peak area ratio calculation for the Raman bands.

The analytical method presented several important advantages. First, the evaluation of the drug content was non-destructive and the assayed microspheres could be used for further *in vitro* investigations. Second, this information can be obtained rapidly: about 1 min is necessary to record a Raman spectrum and the computer-assisted mathematical treatment can be almost instantaneous. Compared with more conventional methods, including spectrophotometric measurements, the Raman approach brought an additional facility and economy of time due to elimination of extended sample preparation (source of errors): for instance, no accurate weighing operation was required. Compared with certain reported quantitative Raman measurements of drugs within polymeric implants,[14] the advantage of the proposed method was the use of the band peak area instead of intensity and of relative instead of absolute values of the spectral parameters, which eliminated numerous experimental errors and complicated data manipulations. The use of the Raman band of the polymer as an internal intensity standard made the measurements completely independent of the overall intensity of the Raman spectra. Hence control of the laser power, focusing and other instrumental conditions was unnecessary. The methodology therefore had a more general analytical character, *i.e.*, not related to particular experimental conditions (instrumental parameters, polymer content, *etc*.).

Finally, the proposed approach provided reproducible results. The precision of 1% obtained in determining the IdUrd incorporation ratio makes it possible to study the *in vitro* drug release kinetics by assaying, by Raman spectroscopy, the remaining drug amounts in the microspheres as a function of time.

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