JOURNAL OF MATRIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 637–643

A comparative study of the use of colorimetric assays in the assessment of biocompatibility

C.J. CLIFFORD
Institute of Orthopaedics and IRC in Biomedical Materials, Brockley Hill, Stanmore, Middlesex HA7 4LP, UK

S. DOWNES
Department of Human Morphology, The Medical School, Queen's Medical Centre, Nottingham, NG7 2UH, UK

A panel of colorimetric assays was assessed for sensitivity, reproducibility, and performance in the investigation of the biocompatibility of a representative range of orthopædic biomaterials, using a commercially available human osteosarcoma-derived cell line. The MTT assay was the most sensitive, with a detection limit of 4×10^2 cells per well against background, while the NR assay was the least sensitive, with no colour change until the cell density reached 2×10^4 per well. All of the assays investigated showed a highly significant "edge" effect when within-plate reproducibility was examined; between-plate reproducibility was good for all assays except the MTT assay. When the assays were tested on cells adherent on biomaterials, there was a wide variation in the results obtained; in particular, the MTS assay showed poor reproducibility in the presence of materials. The MTT and BrdU assays both showed sufficient precision to detect cells on two of the materials studied. The study demonstrates that colorimetric assays are potentially useful in biocompatibility assessment but must be fully validated for the application chosen.

1. Introduction

Bone remodelling around a prosthesis in the early post-operative period following joint replacement is crucial to the long-term stability of the implant, and much research effort has gone into the investigation of this process and the development of biomaterials which will improve it [1–5]. Biocompatibility testing of such materials is vital to ensure that they do not present a risk to the patient and, at the same time, will elicit a biological response.

The human osteosarcoma (HOS) cells used in this study were derived originally from a tumour removed from a 13-year-old female [6]. It has been shown that they produce alkaline phosphatase and osteocalcin [7], both of which are considered to be markers of osteoblast function. Furthermore, Clover and Gowen [8] have demonstrated that they exhibit similar adhesion properties to human osteoblast-like cells; they express the integrin sub-units $\alpha 3$ and $\beta 1$, which can combine to form a receptor which binds to various surfaces. These cells should provide, therefore, a suitable model for the study of the activity of osteoblast-like cells adherent to orthopædic biomaterials.

A range of assays, all of which monitor different aspects of cellular activity, can be applied in the assessment of biocompatibility. First, there are assays based on the use of vital dyes such as Neutral Red and Methylene Blue for the assessment of cell viability. The Neutral Red (NR) assay [9] is based on the spectrophotometric determination of NR (3-amino-*m*-dimethylamino-2-methyl-phenazine hydrochloride) taken up by viable cells and stored in their lysosomes. Methylene Blue (MB), another vital dye, binds to negatively charged moieties, such as the phosphate groups of nucleic acids and side groups of some proteins, at high pH [10] and hence detects all cells, whether viable or not. These assays have been used by other workers in a variety of applications, from the study of the effects of growth factors on adherent and non-adherent cells [10, 11] to the monitoring of osteoblast-like activity *in vitro* in space [12].

The second group of assays measures the action of intracellular enzymes on tetrazolium salts [13–16]. These are converted by intracellular dehydrogenases to coloured formazans which may or may not be water-soluble. The MTT assay [13] is based on the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), the conversion of which produces an insoluble product; it has been applied in many different research areas [17–20]. The assay has been characterized and modified by a number of workers [21–24]; particular attention has been paid to alternative dissolution agents to the dimethyl sulphoxide used by Mosmann [13]. The MTS assay [15, 16] is a more recent development and is based on another tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS), which, when used with an electron

0957–4530

coupling reagent such as phenozine methosulphate (PMS), produces a soluble product whose absorbance can be measured without a further dissolution step.

The third type of assay measures cell proliferation by determination of the incorporation of nucleoside analogues such as ^{3}H-thymidine and 5-bromo-2′-deoxyuridine (BrdU) into replicating nucleic acids. An enzyme immunoassay to measure lymphoid cell proliferation by quantitation of BrdU incorporated into DNA was developed by Porstmann and co-workers [25]. The assay has been used on fixed microcultures [26] which permit further investigation of cell morphology and cell number and may be useful in biocompatibility testing, but the major use of BrdU in recent years has been as a fluorescent label in flow cytometry measurement.

The application of these assays in the monitoring of the interaction between cells and biomaterials presents specific problems. First, the size and shape of the biomaterial samples to be tested may not be uniform. Also most biomaterials have rough surfaces and this will influence the degree of cell adhesion, due to variations in total surface area presented and cell–surface interaction. It is important, therefore, that the assays used are reproducible so that the degree of experimental variability is kept to a minimum. Secondly, cells are likely to adhere to test materials in low numbers so it is essential that the assays used are sufficiently sensitive.

Furthermore, there may be a physical or chemical interaction between the material and the test reagents which, if appropriate controls are not used, will result in 'false positive' results for some materials. Other workers have attempted to bypass these problems by incubating cells with material extracts, prepared by mixing material samples in an appropriate buffer, and then performing the assays but it was decided that, for the purposes of this study, the chosen assays should be carried out on cells interacting with biomaterials *in situ*.

2. Materials and methods

Tissue culture media, additives and Thermanox™ discs were supplied by Life Technologies Ltd, Paisley, UK and plasticware by Becton–Dickinson, Oxford, UK; the materials used were supplied by Goodfellows, Cambridge, UK with the exception of the ceramic, H72, which was donated by Professor Dr Ferdinand Driessens of the Universitat Politecnica de Catalunya in Spain. Trypsin, phosphate-buffered saline (PBS), ascorbate and MTT were supplied by Sigma Chemical Co., Poole, UK. All other reagents were supplied by Merck Ltd, Poole, UK.

The study was carried out using human osteosarcoma (HOS) cells (European Collection of Animal Cell Cultures number 87070202) which were grown to near confluence before being harvested using 0.02% trypsin in phosphate-buffered saline (PBS) buffered with 0.01 M HEPES. The cells were resuspended to the required density in Dulbecco's Modified Eagle's Medium (DMEM), with added foetal calf serum (FCS, 10%), ascorbate (150 μg/ml), L-glutamine (0.02 M), HEPES (0.01 M), non-essential amino acids (1%), penicillin (100 units/ml) and streptomycin (100 μg/ml), and used to seed tissue culture plates as described for individual experiments.

2.1. The assays investigated

2.1.1. MTT assay

A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) in warmed PBS was prepared and filtered before use. It was added to all wells of an assay to give a final concentration of 0.5 mg/ml and the plates were incubated at 37 °C, 5% CO_2 for 4 h. Excess medium and MTT were removed and dimethyl sulphoxide (DMSO) was added to all wells. The plates were mixed for 10 min to ensure crystal dissolution and the absorbance measured on a Dynatech MR700 plate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

2.1.2. MB assay

Adherent cells were washed with 0.15 M sodium chloride and fixed in 10% formal saline at room temperature for 30 min. The fixative was replaced with Methylene Blue (MB, 1% w/v in 0.01 M borate buffer pH 8.5, filtered) and the plates incubated at room temperature for 30 min. Excess dye was removed and the plates washed four times with borate buffer. Ethanol/0.1 M hydrochloric acid (1/1 v/v) was added to solubilize the dye, the plates were mixed and the absorbance measured at 655 nm on a Biorad Model 3550 plate reader.

2.1.3. MTS assay

The cells used were incubated in DMEM without phenol red indicator as this interferes with the assay. The assay was carried out using the CellTiter 96™ Non-Radioactive Cell Proliferation Assay kit available from Promega (Southampton, UK). At the end of the adherence period, a solution of MTS (final assay concentration 333 μg/ml) and PMS (final assay concentration 25 μM) was added to the incubation medium; the plates were mixed and incubated at 37 °C, 5% CO_2 for 2 h. The plates were mixed again and the final absorbance measured at 490 nm on a Biorad Model 3550 plate reader.

2.1.4. Neutral Red assay

Adherent cells were incubated in complete DMEM containing Neutral Red (NR, 50 μg/ml) for 3 h at 37 °C, 5% CO_2. Excess medium was removed and the cells were washed in formaldehyde (0.5%)/calcium chloride (1%). Acetic acid (1%)/ethanol (50%) was added to extract the dye and the plates incubated at room temperature for 30 min. The plates were mixed for 30 min and the absorbance measured at 540 nm on a Biorad Model 3550 plate reader.

2.1.5. Bromodeoxyuridine assay

The assay was carried out on adherent cells using the RPN 210 Cell Proliferation Assay supplied by Amersham International plc (Amersham, UK). The cells were labelled with BrdU for 2 h at 37 °C, 5% CO_2; they were then washed with PBS for 30 s and fixed in 5% acetic acid/90% ethanol for 30 min. All subsequent incubations were at room temperature. The fixed cultures were washed three times in PBS containing 0.1% Tween 20™ (PBS/Tween); blocking agent (PBS/Tween containing 3% bovine serum albumin) was added and the cells incubated for 15 min. The blocking agent was replaced with a solution of nuclease (to facilitate antibody access by DNA denaturation) containing a mouse monoclonal antibody to BrdU and the cultures incubated for 1 h. They were washed in PBS/Tween as before; a peroxidase-linked anti-mouse IgG_{2a} antibody was added and incubated for 30 min. The cultures were washed in PBS/Tween as before and peroxidase substrate was added; the cells were incubated for 30 min before 2 M phosphoric acid was added to stop the enzyme reaction. The final absorbance was measured at 410 nm on a Dynatech MR700 plate reader with a reference wavelength of 630 nm.

2.2. Investigation of the sensitivity and reproducibility of the assays

2.2.1. Sensitivity

A suspension of HOS cells was prepared and used to seed a set of microtitre plates (one for each assay) with a range of numbers of cells from 0 to 2×10^4 per well. The plates were incubated at 37 °C, 5% CO_2 for 24 h to allow cell adherence before the assays were carried out as described.

The sensitivity of each method was assessed by calculating the mean absorbance ± 2 sd for each cell number and determining the limits of detection from control wells without cells and from wells containing 100 cells.

2.2.2. Reproducibility

A HOS cell suspension was prepared and used to seed a set of microtitre plates (two for each assay) with 10^4 cells per well. The cells were incubated and the assays carried out as before.

Within-plate reproducibility was assessed by comparing the mean absorbances in the wells on the outside edges and a set of 36 wells from the central area of the plates, using Student's *t*-test for unpaired data.

Between-plate reproducibility for each assay was assessed by comparing the mean absorbances in sets of 36 wells from the central areas of each pair of plates, using Student's *t*-test for unpaired data.

2.3. Performance of the assays in assessment of the biocompatibility of materials

A panel of materials was selected to represent the range of biomaterials currently used in orthopædic surgery. They were:

1. Polyhydroxybutyrate (PHB) 1 cm^2
2. Titanium foil (Ti) 1 cm^2
3. Ceramic H72 1 cm^2
4. Polystyrene (PS) 1 cm^2
5. Polyethylene (PE) UHMW 1 cm^2
6. Polymethylmethacrylate (PMMA) 1 cm^2

In addition, tissue culture plastic and Thermanox™ discs (15 mm diameter) were used as positive controls.

A total of eight test replicates (incubated with cells) and five (three only for ceramic H72) control replicates (incubated with medium alone) were assayed over three separate experiments. The materials were placed in 24-well tissue culture plates; all wells were sterilised by washing with 0.5 ml absolute ethanol, followed by 1 ml complete DMEM. One set of five plates was seeded with 2×10^4 cells per well and the other with complete DMEM alone to investigate the reaction of the materials themselves in the assays. All plates were incubated at 37 °C, 5% CO_2 for 24 h. The medium was removed and the materials transferred to fresh 24-well plates where the assays were carried out as before. On completion of the assays, aliquots of the final reaction mixture were transferred to 96-well plates for absorbance measurement.

3. Results

3.1. Assay sensitivity and reproducibility

3.1.1. Sensitivity

Figs 1 and 2 show assay standard curves from 0 to 2×10^4 and 0 to 2×10^3 cells per well respectively;

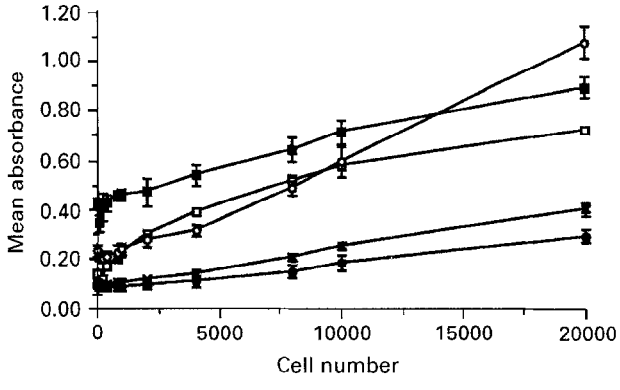

Figure 1 Standard curves for all assays; wells seeded with 0–20000 cells/well: □ MTT; ○ MB; ■ MTS; ● NR; ⋈ BrdU.

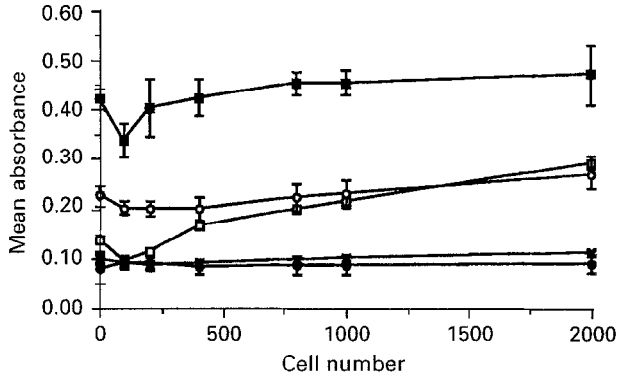

Figure 2 Standard curves for all assays; wells seeded with 0–2000 cells/well: □ MTT; ○ MB; ■ MTS; ● NR; ⋈ BrdU.

TABLE I Detection limits of the assays (assessed by calculating the mean absorbance ± 2 sd for each cell number and determining the limits of detection from control wells without cells and from wells containing 100 cells)

Assay	Detection limit from 0 cells	Detection limit from 100 cells
MTT	400 cells	800 cells
MTS	2000 cells	8000 cells
MB	8000 cells	8000 cells
NR	20000 cells	20000 cells
BrdU	8000 cells	4000 cells

TABLE II Assay reproducibility within plate (data combined from two plates for each assay to calculate mean absorbances ± sd)

Assay	Absorbance in outer wells (mean ± sd)	Absorbance in centre wells (mean ± sd)	p
MTT	0.56 ± 0.02	0.53 ± 0.03	< 0.005
MTS	0.97 ± 0.03	1.01 ± 0.03	< 0.005
MB	0.39 ± 0.05	0.43 ± 0.03	< 0.005
NR	0.10 ± 0.02	0.12 ± 0.01	< 0.005
BrdU	0.17 ± 0.03	0.14 ± 0.01	< 0.005

TABLE III Assay reproducibility between plates (absorbance data from centre wells of two plates for each assay used to calculate mean ± sd)

Assay	Plate 1 absorbance (mean ± sd)	Plate 2 absorbance (mean ± sd)	p
MTT	0.54 ± 0.02	0.52 ± 0.03	< 0.005
MTS	1.01 ± 0.03	1.01 ± 0.03	not significant
MB	0.42 ± 0.03	0.44 ± 0.03	< 0.05
NR	0.12 ± 0.01	0.12 ± 0.01	not significant
BrdU	0.14 ± 0.01	0.14 ± 0.01	not significant

Table I shows the detection limits of the assays. The MB assay shows linearity over the greatest range but its detection limit is 8×10^3 cells per well. The MTT assay showed some linearity at very low cell numbers (Fig. 1b) and is the most sensitive to cell number; it can detect cell numbers as low as 4×10^2 per well against assay background. The NR assay was the least sensitive, with no detectable colour change until the seeded cell number reached 2×10^4 per well.

3.1.2. Reproducibility

None of the assays showed good within-plate reproducibility (Table II); there was a highly significant 'edge' effect ($p < 0.005$) in all cases. However, between-plate reproducibility (Table III) was good for all assays except the MTT assay, which showed a highly significant ($p < 0.005$) difference in the mean absorbance readings from the two plates.

3.2. Assessment of material biocompatibility

Figs 3–7 show the mean absorbance readings obtained when the assays were carried out on control (unseeded) and test (seeded) materials. The absorbance data is shown in Table IV.

The NR assay, though precise, gave absorbance values that were too low to detect any difference between control and test samples. The MB assay, while the absorbance readings obtained were also low, was sufficiently precise to detect the presence of cells

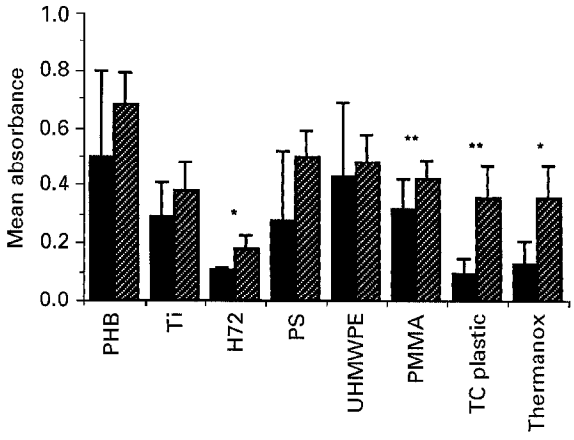

Figure 3 Mean absorbance values in MTT assay from seeded (▩) and unseeded (■) materials. Significant differences indicated by $^{**}p < 0.005$ or $^{*}p < 0.05$.

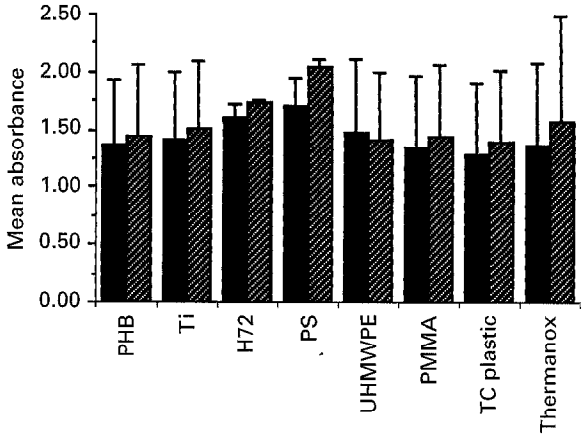

Figure 4 Mean absorbance values in MTS assay from seeded (▩) and unseeded (■) materials. No significant differences observed.

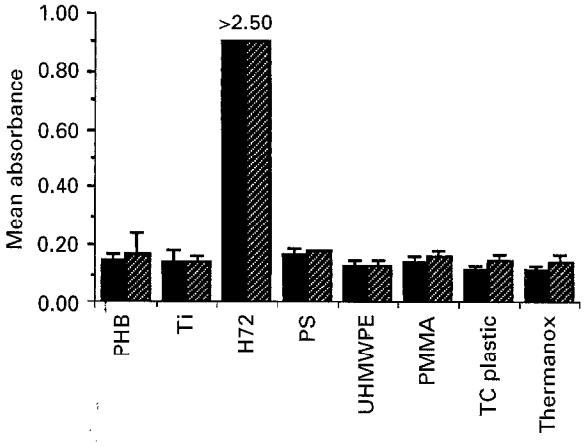

Figure 5 Mean absorbance values in MB assay from seeded (▩) and unseeded (■) materials. Significant differences indicated by $^{*}p < 0.05$.

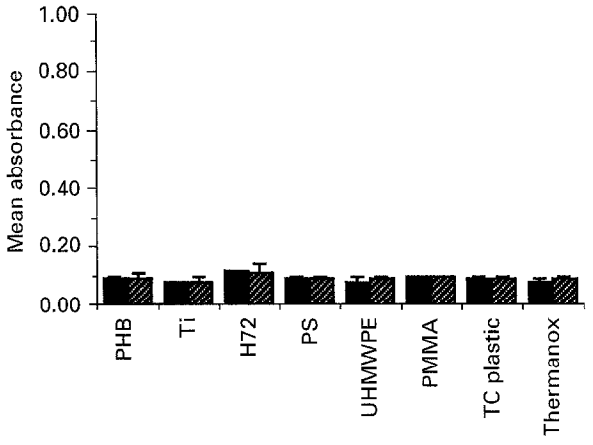

Figure 6 Mean absorbance values in NR assay from seeded (▩) and unseeded (■) materials. No significant differences observed.

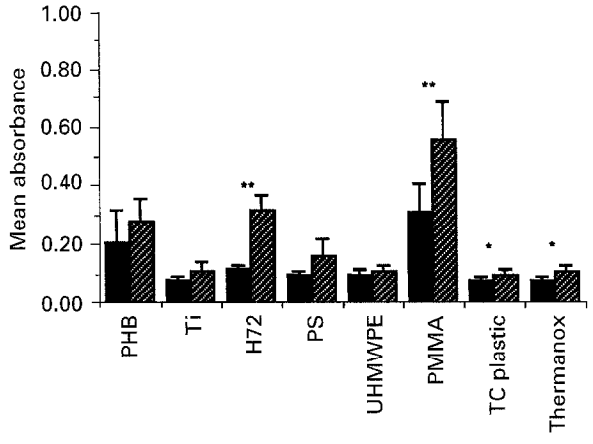

Figure 7 Mean absorbance values in BrdU assay from seeded (▩) and unseeded (■) materials. Significant differences indicated by $^{**}p < 0.005$ or $^{*}p < 0.05$.

on tissue culture plastic; however, in the case of the ceramic H72, visual examination of the material and the absorbance values obtained showed that the dye adsorbed on to the material and was released on addition of acidified ethanol. Significant absorbance values were also obtained in the absence of cells in the MTT assay from PHB, PS, UHMWPE and PMMA and reaction between the assay reagents and certain materials was seen in the BrdU assay (see Table IV).

The MTS assay showed poor inter-experimental precision in the presence of the materials and hence could not detect any significant differences between the test and control materials.

The MTT and BrdU assays gave the most useful results overall. Both gave sufficient precision to detect significant differences between control and test samples of four of the materials in the panel; the differences were highly significant ($p < 0.005$) for the H72 and PMMA.

Fig. 8 shows the absorbance data for all assays on all materials; here the absorbance values for the test materials have been corrected for the values obtained from control materials. There is no overall trend in the results obtained for the panel of materials.

TABLE IV Absorbance data obtained from HOS cells incubated on biomaterials $n = 8$ for test materials, $n = 5$ for control materials ($n = 3$ for H72) and the data was obtained from three separate experiments

Material	MTT		MTS		MB		NR		BrdU	
	Control	With cells	Control	With cells	Control	With cells	Control	With cells	Control	With cells
PHB	0.50 ± 0.30	0.68 ± 0.11	1.37 ± 0.56	1.44 ± 0.62	0.15 ± 0.02	0.17 ± 0.07	0.09 ± 0.01	0.09 ± 0.02	0.21 ± 0.11	0.28 ± 0.08
	p not significant		*p* not significant		*p* not significant		*p* not significant		*p* not significant	
Ti	0.29 ± 0.12	0.38 ± 0.10	1.42 ± 0.58	1.51 ± 0.58	0.14 ± 0.04	0.14 ± 0.02	0.08 ± 0.00	0.08 ± 0.02	0.08 ± 0.01	0.11 ± 0.03
	p not significant		*p* not significant		*p* not significant		*p* not significant		*p* not significant	
H72	0.11 ± 0.01	0.18 ± 0.05	1.60 ± 0.12	1.73 ± 0.03	> 2.50	> 2.50	0.12 ± 0.00	0.11 ± 0.03	0.12 ± 0.01	0.32 ± 0.05
	p not significant		*p* not significant		—		*p* not significant		$p < 0.005$	
TC plastic	0.10 ± 0.05	0.36 ± 0.11	1.28 ± 0.62	1.39 ± 0.62	0.12 ± 0.01	0.15 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.02
	$p < 0.005$		*p* not significant		$p < 0.05$		*p* not significant		$p < 0.05$	
PS	0.28 ± 0.24	0.50 ± 0.09	1.71 ± 0.24	2.04 ± 0.07	0.17 ± 0.02	0.18 ± 0.00	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.16 ± 0.06
	p not significant		*p* not significant		*p* not significant		*p* not significant		*p* not significant	
UHMWPE	0.43 ± 0.26	0.48 ± 0.10	1.48 ± 0.63	1.42 ± 0.58	0.13 ± 0.02	0.13 ± 0.02	0.08 ± 0.02	0.09 ± 0.01	0.10 ± 0.02	0.11 ± 0.02
	p not significant		*p* not significant		*p* not significant		*p* not significant		*p* not significant	
PMMA	0.32 ± 0.10	0.42 ± 0.07	1.34 ± 0.62	1.44 ± 0.62	0.14 ± 0.02	0.16 ± 0.02	0.10 ± 0.00	0.10 ± 0.00	0.31 ± 0.10	0.56 ± 0.13
	$p < 0.05$		*p* not significant		*p* not significant		*p* not significant		$p < 0.005$	
Thermanox	0.13 ± 0.08	0.36 ± 0.11	1.37 ± 0.71	1.58 ± 0.91	0.12 ± 0.01	0.14 ± 0.03	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.11 ± 0.02
	$p < 0.005$		*p* not significant		*p* not significant		*p* not significant		$p < 0.05$	

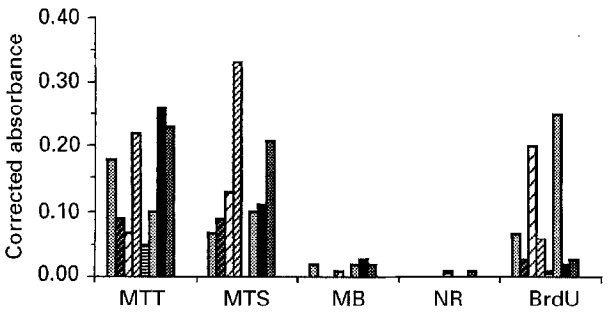

Figure 8 Mean absorbance values obtained in assays on adherent cells, corrected for reaction of materials with assay reagents: ▨ PHB; ▩ Ti; ▨ H72; ▨ PS; ▤ UHMWPE; ▩ PMMA; ■ TC plastic; ▩ Thermanox™.

4. Discussion

The assays showed considerable variation in performance when used to determine the viability and proliferation of the human osteosarcoma cell line used. The MTT, MTS and BrdU assays have open-ended incubation times which may account for this to some extent. The MB assay gave the highest absorbance values but this was to be expected since, of the assays tested, it is the only one which will detect viable and non-viable cells [10]. Elliott and Auersperg [27] used human ovarian cells to compare the MB and NR assays and demonstrated that the MB assay is more sensitive than the NR assay; the findings reported here, using a different cell line, confirm their results. The NR assay gave the lowest absorbance values and, although reproducible, was the least sensitive; Zhang *et al.* [28] showed that NR uptake increased as the cells aged and lysosomes proliferated suggesting that the NR assay would have performed better if the cultures had been incubated for longer than 24 48 h. This will have to be examined.

The MTT and MTS assays gave widely differing results in this study; this was somewhat surprising in view of the fact that the two compounds are essentially slightly different substrates for the same intracellular enzymes and therefore might be expected to give parallel results. In fact, in our hands, the MTS assay was less sensitive than the MTT assay in terms of cell detection although it showed better reproducibility between plates. The MTT assay, on the other hand, was by far the most sensitive assay of those investigated although it was not as reproducible as some of the others in the between-plate studies. Wan *et al.* [24] investigated the reproducibility of the MTT assay in some depth and demonstrated that its reproducibility was not good; they suggested that this was due to variations in cell number and it is possible that, in the plates used in our study, there were differences in the numbers of cells actually adhering in the individual wells which gave rise to the variability seen.

The BrdU assay was not very sensitive although its reproducibility was acceptable; of the assays studied it was the only one that measured true cell proliferation and the low absorbance values seen may have been due to the short-term nature of the experiments. Muir *et al.* [26] established monolayer cultures of Schwann cells for 24 h before stimulating proliferation for 16 h and allowing BrdU incorporation to take place for a further 16 h which suggests that, like the NR assay, the BrdU assay performs better in longer-term cultures.

The most important observation reported in this paper is the lack of an overall trend in the results obtained when the panel of assays was used to measure the activity of cells cultured on biomaterials used in orthopædics. All the assays were applied to the materials alone and there was some evidence of reaction between the assay reagents and the materials, particularly in the case of MB and the ceramic H72, which was extreme. The only assays which showed parallel results at all were the MTT and BrdU assays, which both detected cellular activity on the control materials, TC plastic and Thermanox; these two assays also suggested that there was significant activity and proliferation on PMMA.

Biocompatibility has been defined previously as "the ability of a material to perform with an appropriate host response in a specific application" [29] and the consideration of what constitutes an "appropriate host response" in the orthopædic situation will determine the selection of assays to assess the biocompatibility of new and existing materials. This study demonstrates that some colorimetric assays do have potential for use in the direct assessment of biocompatibility of materials but that they should be fully validated for the cells and materials to be studied before any conclusions can be drawn.

Acknowledgements

This work forms part of the DTI supported AM2 project (Advanced Materials Programme) "The development of test methods to enable the generation of a materials biocompatibility index".

References

1. S. DOWNES, L. DI SILVIO, C. P. A. T. KLEIN and M. V. KAYSER, *J. Mater. Sci. Mater. Med.* **2** (1991) 176.
2. K. SØBALLE, E. HANSEN, H. BROCKSTEDT-RASMUSSEN and C. BUNGER, *J. Bone Joint Surg.* **75B** (1993) 270–278
3. H. WEINANS, R. HUISKES and H. GROOTENBOER, *J. Orthop. Res.* **10** (1992) 845.
4. S. DOWNES, M. BRADEN, R.S. ARCHER, M. PATEL, K. DAVY and H. SWAI, in "Surface properties of biomaterials" (Butterworth-Heinemann, Oxford, 1994) p. 11.
5. L. DI SILVIO, M. KAYSER and S. DOWNES, *Clin. Mater.* **16** (1994) 91.
6. R. M. McALLISTER, M. B. GARDNER, A. E. GREENE, C. BRADT, W. W. NICHOLS and B. H. LANDING, *Cancer* **27** (1971) 397.
7. L. DI SILVIO, PhD thesis, University of London (1995) p. 46.
8. J. CLOVER and M. GOWEN, *Bone* **15** (1994) 585.
9. E. BORENFREUND and J. PUERNER, *Toxicol. Lett.* **24** (1985) 119.
10. M. H. OLIVER, N. K. HARRISON, J. E. BISHOP, P. J. COLE and G. J. LAURENT, *J. Cell Sci.* **92** (1989) 513.
11. C. LOWIK, M. ALBALS, M. van de RUIT, S. PAPAPOULOS and G. van der PLUIJM, *Anal. Biochem.* **213** (1993) 426.
12. C. GENTY, S. PALLE, L. VANELLE, S. BOURRIN and C. ALEXANDRE, *Biotech. Histochem.* **69** (1994) 160.
13. T. MOSMANN, *J. Immunol. Meth.* **65** (1983) 55.
14. N. ROEHM, G. RODGERS, S. HATFIELD and A. GLASEBROOK, *ibid.* **142** (1991) 257.

15. A. CORY, T. OWEN, J. BARLTROP and J. CORY, *Cancer Commun.* **3** (1991) 207.
16. T. RISS and R. MORAVEC, *Mol. Biol. Cell.* **3(Suppl)** (1992) 184a.
17. K. BERG, M. B. HANSEN and S. E. NIELSEN, *APMIS* **98** (1990) 156.
18. C.-H. CHEN, P. A. CAMPBELL and L. S. NEWMAN, *Int. Arch. Allergy Appl. Immunol.* **93** (1990) 249.
19. G. CIAPETTI, E. CENNI, L. PRATELLI and A. PIZZOFERRATO, *Biomaterials* **14** (1993) 359.
20. D. A. MONNER, *Immunol. Lett.* **19** (1988) 261.
21. F. DENIZOT and R. LANG, *J. Immunol. Meth.* **89** (1986) 271.
22. J. A. PLUMB, R. MILROY and S. B. KAYE, *Cancer Res.* **49** (1989) 4435.
23. D. T. VISTICA, P. SKEHAN, D. SCUDIERO, A. MONKS, A. PITTMAN and M. R. BOYD, *ibid.* **51** (1991) 2515.
24. H. WAN, R. WILLIAMS, P. DOHERTY and D. F. WILLIAMS, *J. Mater. Sci. Mater. Med.* **5** (1994) 154.
25. T. PORSTMANN, T. TERNYNCK and S. AVRAMEAS, *J. Immunol. Meth.* **82** (1985) 169.
26. D. MUIR, S. VARON and M. MANTHORPE, *Anal. Biochem.* **185** (1990) 377.
27. W. ELLIOTT and N. AUERSBERG, *Biotech. Histochem.* **68** (1993) 29.
28. S.-Z. ZHANG, M. LIPSKY, B. TRUMP and I.-C. HSU, *Cell Biol Toxicol.* **6** (1990) 219.
29. D. F. WILLIAMS, *J. Biomed. Eng.* **11** (1989) 185.

Received 23 June
and accepted 21 December 1995