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# Methods for the evaluation of biocompatibility of soluble synthetic polymers which have potential for biomedical use: 1 – Use of the tetrazolium-based colorimetric assay (MTT) as a preliminary screen for evaluation of *in vitro* cytotoxicity

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A tetrazolium-based colorimetric assay (MTT) was first introduced by Mossman in 1983 to assess the potential of novel antitumour agents, and it has been used here to evaluate the cytotoxicity of several soluble synthetic polymers proposed as drug carriers. Polymers including poly-L-lysine (molecular weight 57 000) were incubated (up to 1 mg ml$^{-1}$) with two human cell lines, hepatocellular carcinoma ($HepG_2$) and lymphoblastoid leukaemia (CCRF), adherent and suspension cells, respectively. Tests were carried out in the presence and absence of serum proteins. The assay was first modified to optimize the colorimetric profiles produced by the cell lines following incubation with MTT, to increase both the test sensitivity and the reproducibility of the method. Polymer toxicity observed using the MTT test was compared with data obtained using other methods; [$^3$H]thymidine or [$^3$H]leucine incorporation and counting cell numbers. Poly-L-lysine was very toxic to both cell lines with approximate $IC_{50}$-values of 60 and 30 $\mu$g ml$^{-1}$ for $HepG_2$ and CCRF, respectively, the values obtained being similar for each of the three different viability methods used. In the absence of serum proteins the toxicity of poly-L-lysine increased, the $IC_{50}$-values falling to 25.5 $\mu$g ml$^{-1}$ for the adherent and 0.8 $\mu$g ml$^{-1}$ for the suspension cell line. Other polymers such as poly-L-proline, polyethylene glycol, dextran, polyvinylpyrrolidone and poly-L-glutamic acid were not cytotoxic (MTT assay), either in the presence or in the absence of serum proteins. The MTT assay is a useful technique for the primary and rapid evaluation of the cytotoxicity of soluble polymers.

## 1. Introduction

Soluble synthetic polymers have many potential uses in medicine, including development as plasma expanders, haemoglobin substitutes and as drug-carrier systems. As novel soluble synthetic polymers are synthesized their suitability for human use, "biocompatibility", must be addressed. Biocompatibility has been defined previously as "the ability of a material to perform, with an appropriate host response in a specific application" [1]. For specific biomedical applications an ideal polymer would be non-toxic, non-immunogenic, biodegradable and, if used as a drug carrier, would also have a high drug-carrying capacity, the ability to deliver drug selectively and control its rate of release at the target site [2].

Although biocompatibility encompasses many properties of a material, an important aspect of prescreening new polymers of biomedical potential is to establish their general cytotoxicity to cells cultured *in vitro*. Compounds which are highly (and non-specifically) toxic will never be suitable for *in vivo* use. Due to their relatively recent development for medical use, there are no guidelines for cytotoxicity testing of soluble polymers. However, protocols are available for the evaluation of novel implant materials [3].

To develop *in vitro* methods for screening the toxicity of soluble polymers, studies should ideally be undertaken using widely available established human cell lines, and for a rational design of the testing procedure the particular cell lines selected should depend on the material application, route of administration and expected organ toxicity (if predictable). In recent years we have been trying to identify a portfolio of tests which could be used for the primary evaluation of the biocompatibility of novel soluble polymers. Preliminary data have been presented in abstract form describing routine methods for evaluation of haematoxicity and immunogenicity [4, 5].

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The main aim of this study was to evaluate, using two human cell lines (an adherent cell line (hepatoma, $HepG_2$) and a suspension culture (lymphoblastoid leukaemia, CCRF)), the suitability of the tetrazolium dye-based colorimetric assay (MTT) as a preliminary and rapid method for the assessment of the cytotoxicity of soluble polymers. This test is advantageous, as it can be carried out quickly as a microtitre plate assay. Traditionally the reduction of the tetrazolium salts to their equivalent formazan precipitates was used for the histochemical demonstration of activity of oxidative and non-oxidative enzymes in mitochondria using both light and electron microscopy [6]. In 1983 Mossman [7] developed the assay to assess the effects of proliferative lymphokines, mitogen stimulation and complement-mediated lysis. More recently the assay has been developed extensively as a quick and effective screening method for the assessment of mitochondrial impairment by potentially cytotoxic novel antitumour agents [10]. Several assay-related problems have been described, particularly inadequate levels of formazan generation by some cell lines, limited solubility and stability of the MTT formazan solutions in organic solvent systems [8–10]. However, these problems can be largely overcome by use of spectrophotometric grade dimethylsulphoxide (DMSO) which provides absorbance stability for up to 2 h [8] and addition of sodium succinate to enhance the reaction [11].

Several polymers, which have been studied previously for specific biomedical applications [12–16], were chosen to standardize the assay, including poly-L-glutamic acid (PGA), poly-L-lysine (PLL), polyvinylpyrrolidene (PVP), polyethylene glycol (PEG) and dextran (DEX), and their characteristics are shown in Table I. Polymers were incubated with the above cell types continuously for 72 h at concentrations ranging from 0.5 $\mu g\,ml^{-1}$ to 1 $mg\,ml^{-1}$. The effect on cell growth and viability was determined using the MTT assay, and the results obtained are compared with those derived from other techniques used for routine analysis of the viability or growth of cell cultures (incorporation of radiolabelled nucleotides or amino acids, and measurement of cell numbers). Experiments were conducted in the presence and absence of serum proteins.

## 2. Materials and methods

### 2.1. Cell lines

Human $HepG_2$ cells [17] were grown as a monolayer culture in minimum essential medium (MEM) supplemented by 10% foetal bovine serum. They were subcultured once a week at a 1 : 5 split ratio, (washing with Trypsin–ethylenediaminetetraacetic acid (EDTA) to detach the cells). In addition the medium was replaced every 3 to 4 days to avoid depletion of essential nutrients. Human CCRF cells [18] were grown as a suspension culture in RPMI medium supplemented with 10% foetal bovine serum. They were subcultured once a week at a 1 : 10 split ratio. Growth curves were established for both of the cell lines, cultured in flasks, tubes and/or in microtitre plates, and the timing of the experiments was chosen to ensure exposure of cells to the polymer during the logarithmic phase of their growth (Fig. 1). No antibiotics were used in the cell cultures and all cells were mycoplasma-free. Culture media and sera were supplied by Flow Laboratories.

### 2.2. Polymers

The polymers used are listed in Table I and were supplied by Sigma Chemical Co., Poole, UK, with the exception of PEG, which was supplied by Polysciences Inc., Warrington, USA. MTT and DMSO (spectrophotometric) grade were also supplied by Sigma Chemical Co.

### 2.3. *In vitro* cytotoxicity: general methods

Cells were incubated with polymers in 96-well microtitre plates, thus enabling testing of a wide range of polymer concentrations (with sufficient replication for statistical analysis), with the use of minimal amounts of material. CCRF cells were plated into each well at a density of 4000 cells $ml^{-1}$ (determined by a Coulter Counter®). The seeding density of $HepG_2$ was approximately (4 to 6) $\times$ $10^4$ cells $ml^{-1}$ (the exact number could not be precisely quantified, because these cells tend to form aggregates which prevent reliable use of a Coulter Counter or a haemocytometer slide). Subsequently, the cells were left to recover and enter the logarithmic phase of growth (24 h for the $HepG_2$ and 4 days for the CCRF). When experiments were carried out in the absence of serum, no initial cell equilibration period was possible in the case of CCRF cells, due to their reduced lifespan, and thus they were plated at a density of 4 $\times$ $10^5$ cells $ml^{-1}$. The $HepG_2$ cells were washed twice with PBS before the addition of the polymers, to ensure complete removal of the serum proteins.

Polymers were added to microtitre plates in a volume of 50 $\mu l$ to give a total volume of 250 $\mu l$. The polymer solution was prepared in the appropriate tissue culture medium without the addition of serum

TABLE I Polymers and their chemical characteristics

| Polymer | Abbreviation | Molecular weight* | Degree of polymerization | pH† |
|---|---|---|---|---|
| Poly-L-lysine | PLL | 57 000 | 270 | 7.75 |
| Poly-L-glutamic acid | PGA | 26 500 | 175 | 7.49 |
| Poly-L-proline | PLP | 54 000 | 554 | 7.62 |
| Poly-L-proline | PLP | 8000 | 78 | 7.60 |
| Dextran | DEX | 40 200 | NA | 7.52 |
| Polyethylene glycol | PEG | 8000 | NA | 7.56 |
| Polyvinylpyrrolidone | PVP | 40 000 | NA | 7.56 |

*Weight average molecular weight: information provided by the supplier, determined by LALLS or viscometry.
†1 $mg\,ml^{-1}$ polymer solution in minimum essential medium.
NA, information not available from the supplier.

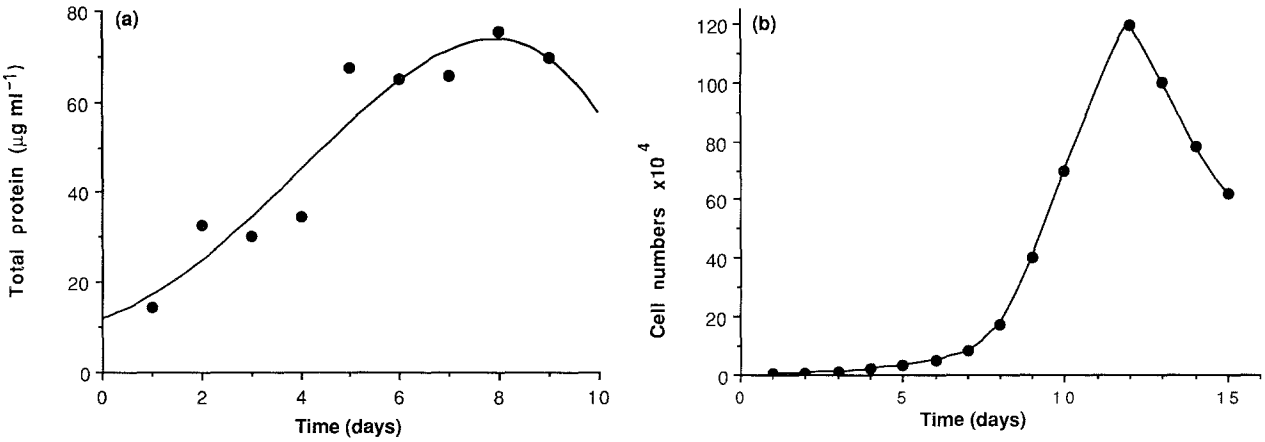


*Figure 1* Growth curve for (a) $HepG_2$ adherent cell line measured by protein estimation (the Lowry assay) and (b) CCRF suspension cell line measured by cell counting.

proteins, to minimize the chance of aggregate formation between the charged sites of proteins and polymers. Before use all solutions were sterilized through a Flowpore D filter (0.2 μm). To overcome the potential problem of polymer adherence to the filters, they were first saturated with a small portion of the appropriate polymer solutions. In addition, the pH of the polymer preparations, and that of the culture medium following the addition of the polymers, was routinely measured. In the event of a polymer altering pH away from neutrality, further dilutions in medium were made to ensure that tests were always carried out at pH 7.4, therefore excluding false-positive cytotoxicity results as a simple effect of the pH.

After the addition of polymers to the microtitre plates, cells were incubated for 12 to 72 h, at the end of which time viability tests were performed. Cells incubated in the absence of polymer (untreated controls) and microtitre plate wells containing medium but no cells (background blanks) were used as a reference.

## 2.4. The MTT assay

The assay is based on the observation that viable cells have the ability to metabolize a water-soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), into an insoluble formazan salt [6, 7]. MTT in PBS (20 μl well$^{-1}$ of a 5 mg ml$^{-1}$ MTT solution) was added 5 h before the end of the incubation period. At the end of this time the medium was removed and the insoluble formazan crystals were dissolved in 200 μl DMSO. Finally, the absorbance was measured at 550 nm, using an enzyme-linked immunosorbent assay microtitre plate spectrophotometer. In experiments where the MTT assay was performed on the CCRF suspension culture, before the aspiration of the medium, the cells were centrifuged at 2000 r.p.m. for 10 min to avoid the accidental removal of the formazan crystals. To ensure that the polymers themselves did not contribute to the reduction of the tetrazolium dye, polymer solutions alone were assayed in a microtitre plate by the MTT assay.

In addition, a brief study was carried out to evaluate the effect of the pH on the MTT assay. Serial dilutions of sodium acetate buffer (pH 4.4) in MEM were prepared (pH range 4.5 to 8.5) and assayed by the MTT assay in the presence or absence (controls) of $HepG_2$ cells.

## 2.5. Incorporation of [$^3$H]thymidine (TdR) or [$^3$H]leucine (Leu)

Before the end of the incubation time (24 h for [$^3$H]Leu and 5 h for [$^3$H]TdR), the radiolabelled substances were added (50 μl, 5 μCi) and the incubation completed. Subsequently the cells were harvested, using a cell harvester, and were assayed for radioactivity.

## 2.6. Estimation of cell numbers

CCRF cells were counted using a Coulter Counter. Cells were removed from each well of the microtitre plate and added to a vial containing Isoton® solution to a total volume of 10 ml. Samples of Isoton and cell-free culture medium were also measured.

## 2.7. Analysis

Data obtained following incubation of cells with polymer (MTT assay, cell numbers, isotope incorporation) were expressed as a percentage of the values obtained from untreated control cells within the same experiment. The standard errors of the mean percentages were calculated using a formula derived from [19]:

$$\mathrm{Se} \approx \left[ s_x^2 \left( \frac{1}{\bar{Y}^2} \right) + s_y^2 \left( \frac{\bar{X}^2}{\bar{Y}^4} \right) \right]^{1/2}$$

where Se is the standard error of the ratio $\bar{X}/\bar{Y}$, $\bar{X}$ is the mean percentage viability of the treated cells, $\bar{Y}$ is the mean percentage viability of the untreated cells, $s_x$ is the standard deviation in the calculation of the mean percentage viability $\bar{X}$ of the treated cells and $s_y$ is the standard deviation in the calculation of the mean percentage viability $\bar{Y}$ of the untreated cells.

# 3. Results

Different cell lines do not generally exhibit equal ability to incorporate and metabolize MTT [20]. Both cell lines studied here, $HepG_2$ and CCRF, were able to metabolize the tetrazolium dye MTT to produce optical densities sufficient for the evaluation of toxicity. In the absence of serum proteins (with the same cell density) the absorbance values obtained were much

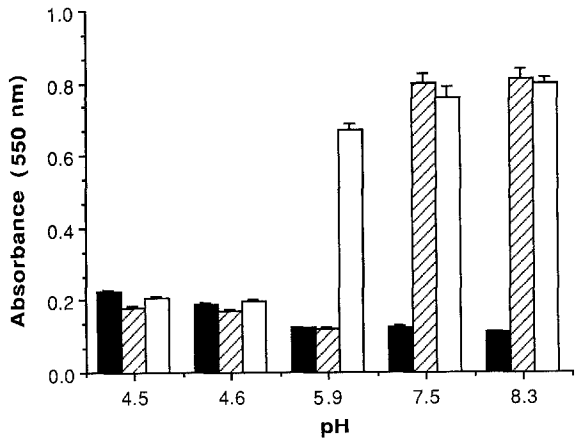


*Figure 2* Effect of incubation pH on the MTT assay ($HepG_2$). The pH values were recorded in the wells before MTT addition. Viability was assayed for: (■) blank (no cells); (▨) cells following 72 h incubation in the appropriate pH (□) cells following 4 h incubation in the appropriate pH.

higher. None of the polymers showed an inherent ability to reduce MTT to formazan.

To study the pH-dependence of the MTT test, $HepG_2$ cells were incubated in serial dilutions of sodium acetate buffer pH 4.5 to 8.3 (pH measured at the end of the incubation). Data obtained are shown in Fig. 2. Cells incubated (briefly or for 72 h) at pH < 5.0 lost all ability to reduce MTT, an observation which is in agreement with the literature [6]. Following 72 h incubation at pH 5.9 cells again lost their ability to reduce MTT, but in this case there was only a small decrease in viability if the incubation period was short (Fig. 2). As can be seen from Table I, the pH values of all stock solutions of the polymer preparations were in the range 7.5 to 7.8. The polymers were also diluted into the cell culture medium, so it is obvious that the medium pH did not contribute to the results observed.

PLL decreased the viability of $HepG_2$ and CCRF cells irrespective of the assay used (Fig. 3), being significantly more toxic to CCRF than $HepG_2$ cells (both in the presence and absence of serum; Fig. 4). The concentration-dependence of toxicity was in both cases non-linear, therefore $IC_{50}$- and $IC_{90}$-values were routinely used to describe these curves. The mean $IC_{50}$-values determined for PLL are given in Table II.

For the $HepG_2$ cell line, the $IC_{50}$-values obtained for the MTT assay and [$^3$H]TdR incorporation were 59.9 ± 10.4 and 61.4 ± 15.6 $\mu g\,ml^{-1}$ (mean ± s.e.m.), respectively. The CCRF cell line showed reproducibly lower $IC_{50}$-values for each test; MTT assay (23.9 ± 5.6 $\mu g\,ml^{-1}$), [$^3$H]TdR incorporation (35.9 ± 3.8 $\mu g\,ml^{-1}$) and estimation of cell numbers (28.7 ± 5.8 $\mu g\,ml^{-1}$). The individual $IC_{50}$-values obtained for the different viability assays, within the same experiment showed excellent correlation (Fig. 5). However, variation in the absolute values obtained was observed between the different experiments (Fig. 6).

For $HepG_2$ the mean $IC_{90}$-value measured for PLL using the MTT assay was 15.2 ± 4.0 $\mu g\,ml^{-1}$ and in this case a similar $IC_{90}$-value was seen for CCRF (11.0 ± 3.0 $\mu g/ml^{-1}$). Evaluation of viability by [$^3$H]TdR incorporation and cell numbers for the CCRF cell line gave reproducible $IC_{90}$-values, 10.9 ± 2.0 and 11.0 ± 3.7 $\mu g\,ml^{-1}$, respectively (Fig. 6).

Incubation in the absence of serum with PLL reduced the $HepG_2$ $IC_{50}$ by 57% and the CCRF $IC_{50}$ by 97% approximately (Table II), emphasizing the differential resistance to lysis of the two cell lines (Fig. 4). The cytotoxic effect of PLL is time-dependent, and occurs faster at higher concentrations. Morphological evaluation of the cultures incubated in the absence of serum revealed complete lysis of CCRF within the first 15 min and for $HepG_2$ within 1 h (results not shown).

PLP did not show any significant cytotoxic effect up to a concentration of 0.5 $mg\,ml^{-1}$ for the $HepG_2$ and the CCRF (data not shown) cell lines, irrespective of the molecular weight.

Similarly, the other polymers investigated (assayed using the MTT test) did not display any cytotoxicity against CCRF or $HepG_2$ at concentrations up to 1 $mg\,ml^{-1}$ and even when experiments were carried out in the absence of serum (Tables III and IV).

## 4. Discussion

The main aim of this study was to examine the potential of the MTT assay for rapid evaluation of cytotoxicity of soluble synthetic polymers *in vitro*. Soluble polymers display variable behaviour in biological systems, depending on their chemical characteristics, mode of synthesis and purification. Factors such as average molecular weight, polydispersity, distribution of charge, residual monomer, nature of polymer (block, random, linear or branched) or conformation have all been shown to determine their biological properties; rates of cell internalization [21], pharmacokinetics *in vivo* [22], degradation [23] and, not least, pharmacological activity [24]. These factors

TABLE II Summary of the $IC_{50}$-values obtained following incubation of PLL with $HepG_2$ or CCRF cells (measured in the presence and absence of serum)

| Cell line | Assay method | Presence of serum | $IC_{50}$ ($\mu g\,ml^{-1}$)* | Number of experiments |
|---|---|---|---|---|
| $HepG_2$ | MTT | + | 59.9 ± 10.4 | 7 |
| $HepG_2$ | MTT | − | 25.5 ± 15.5 | 2 |
| $HepG_2$ | [$^3$H]TdR | + | 61.4 ± 15.6 | 4 |
| CCRF | MTT | + | 23.9 ± 5.6 | 8 |
| CCRF | MTT | − | 0.8 | 1 |
| CCRF | [$^3$H]TdR | + | 35.9 ± 3.8 | 4 |
| CCRF | [$^3$H]TdR | − | 0.8 | 1 |
| CCRF | Cell numbers | + | 28.7 ± 5.8 | 7 |

*The values shown represent the mean ± s.e.m. of the $IC_{50}$-values obtained from the best-fit curve in each experiment.

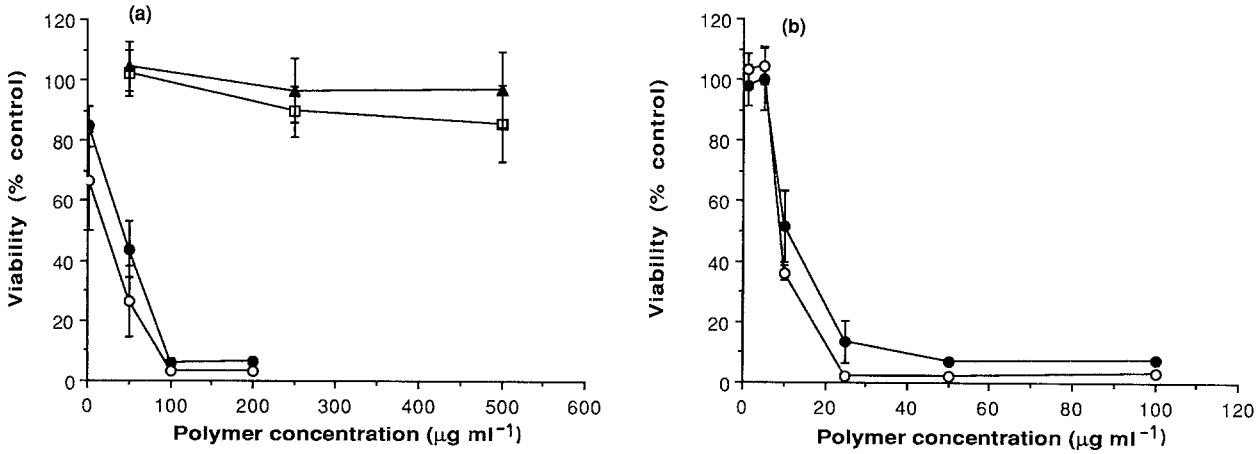


*Figure 3* Effect of PLL and PLP on $HepG_2$ or CCRF cells. (a) $HepG_2$ incubated with PLP of molecular weight (▲) 8000 or (□) 54 000 (effect on viability measured using the MTT assay), or PLL (viability evaluated using (○) the MTT assay or (●) [$^3$H] Leu incorporation). (b) CCRF cells incubated with PLL and viability assayed using (●) the MTT assay or (○) cell numbers.

have also been shown to be important in determining toxicity, particularly immunogenicity [23]. Therefore, to minimize interbatch variation it is important to ensure that standard polymer samples are used throughout.

Certain cellular considerations are also worthy of mention. It is essential that cell cultures are preselected for use in such assays which display a logarithmic growth phase (Fig. 1). For example, the metabolism of MTT is slower once cells reach confluence (adherent cell lines) or at the limiting critical concentration (suspensions) [20]. Similarly, it is always important to ensure that a change in the pH of the incubation medium does not bias the data obtained (Fig. 2). Differences were seen in the behaviour of the adherent and non-adherent cell lines. Incomplete detachment of the $HepG_2$ cells led to greater variation in results obtained when measuring incorporation of [$^3$H]TdR. In contrast, CCRF cells and [$^3$H]TdR incorporation produced a very small variation in the results, which is due to the easier handling of the suspension culture with more-accurate estimation of cell numbers in the initial stages of the experiment. Difficulty in determining the precise seeding density of the $HepG_2$ cultures contributed to increased variation in the results obtained. Higher seeding densities reach confluence and a critical lethal concentration faster, so cytotoxicity may be overestimated.

In addition to the nature of the cell culture, another factor that influences cell behaviour in toxicity assays is the presence of the serum proteins in the culture medium. Serum proteins are important in at least two ways. First, such proteins serve to promote cell growth, provide a supply of nutrients and their coating of the cell surface often increases cell stability and can also protect to some extent against surface-active agents. Secondly, some polymers, including polycations, are known to cause agglutination of proteins [25], and thus interaction with serum proteins in the culture medium could lower the effective medium concentration of the test material.

The MTT assay proved reproducible between and within experiments (Table II), and in fact displayed a lower degree of intra-experimental variation than was seen using other methods frequently used to assess cell viability (Table II). When using cells in culture to evaluate toxicity, the non-linearity of the toxicity–dose response curves (Fig. 3), necessitates selection of specific parameters that can be usefully used for the characterization of the data. We feel that the initial ($IC_{90}$) and the midpoint cytotoxicity concentrations ($IC_{50}$) give a good indication of the kinetics of the response curves (Fig. 6). Despite the fact that the biochemical bases of the viability assays chosen were different, there was a very good correlation between $IC_{50}$- and $IC_{90}$-values obtained using these methods.

TABLE III Cytotoxicity of soluble polymers measured using the MTT assay and CCRF cells in the absence of serum proteins*

| Concentration ($\mu g\,ml^{-1}$) | Viability (% untreated control) | | | | |
|---|---|---|---|---|---|
| | PLL† | PGA | PVP | DEX | PEG |
| 500.0 | 0.2 ± 0.5 | 87.2 ± 13.6 | 98.6 ± 12.8 | 104.3 ± 13.1 | 98.2 ± 11.4 |
| 375.0 | 0.7 ± 2.5 | 94.7 ± 10.9 | 94.8 ± 11.4 | 101.8 ± 18.6 | 98.2 ± 11.4 |
| 250.0 | 1.3 ± 1.7 | 96.1 ± 10.9 | 95.2 ± 13.9 | 102.9 ± 13.8 | 92.9 ± 14.1 |
| 125.0 | 1.4 ± 1.4 | 102.9 ± 13.8 | 97.2 ± 12.7 | 96.5 ± 20.4 | 94.8 ± 11.4 |
| 50.0 | 2.4 ± 0.7 | 99.0 ± 15.8 | 95.8 ± 12.6 | 100.4 ± 15.5 | 96.8 ± 11.1 |
| 25.0 | 1.9 ± 1.5 | 101.4 ± 14.8 | 97.2 ± 11.7 | 98.0 ± 11.9 | 95.8 ± 10.9 |
| 12.5 | 1.4 ± 1.8 | 101.8 ± 11.5 | 96.3 ± 11.5 | 102.9 ± 13.8 | 95.0 ± 11.3 |
| 5.0 | 1.6 ± 1.7 | 102.9 ± 13.8 | 98.2 ± 12.6 | 101.8 ± 14.8 | 99.6 ± 12.4 |
| 2.5 | 2.5 ± 0.8 | 101.8 ± 14.6 | 97.2 ± 14.4 | 102.9 ± 13.5 | 98.6 ± 11.6 |
| 0.5 | 60.2 ± 15.1 | 105.3 ± 14.8 | 100.1 ± 12.5 | | |

*Incubation time 24 h, eight replicates per concentration, values shown are the mean ± s.e.m.
†Abbreviations shown in Table I.

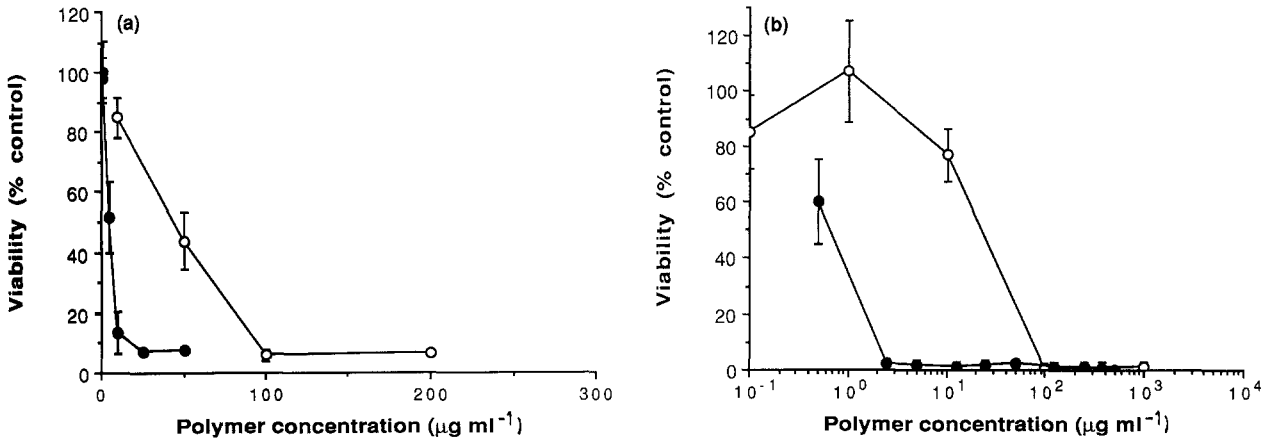


*Figure 4* Comparison of PLL cytotoxicity to $HepG_2$ and CCRF cell lines assessed using the MTT assay. (a) The effect of PLL on (○) $HepG_2$ and (●) CCRF measured in the presence of serum over 72 h incubation. (b) The effect of PLL on (○) $HepG_2$ and (●) CCRF measured in the absence of serum over 24 h incubation.

There was an equally good correlation between the values obtained from the different viability assays within the same experiment. The $IC_{90}$-values obtained were identical for all viability assays used, suggesting that PLL exposure produced no subtle differences in the effect on the electron transport system and nucleic acid synthesis (Fig. 6).

Unlike the other polymers investigated, PLL was reproducibly toxic to both cell lines using all assay methods. However, the suspension culture was much more sensitive than the adherent one, and the presence of serum proteins diminished PLL-induced toxicity. Perhaps hepatocytes were more resistant due to their normal physiological role in detoxification, but more likely they displayed greater tolerance because accessibility of the cytotoxic agent to cells is restricted in a well-structured tightly packed adherent culture. Differences in susceptibility to PLL have been demonstrated before using Erhlich ascites and L1210 mouse leukaemia [26]. The mechanism of PLL toxicity has also been studied previously. Toxicity seems to be related to polycation attachment to the cell surface membrane [27–29] due to ionic interactions with the phospholipids and it is clearly molecular weight-, concentration- and time-dependent [30]. Arnold *et al.* [26] showed that PLL promotes cellular efflux of small molecules, such as $K^+$, inorganic phosphate, carbohydrates, free amino acids, small peptides and adenosine 5′-phosphate, and it is thought that this initial lytic phenomenon could also be linked to phospholipase $A_2$ activation. The observed inhibition of [$^3$H]Leu or [$^3$H]TdR incorporation has been related to polymer binding to the cell membrane, thus reducing either the appropriate recycling capacity of selected membrane components or the membrane fluidity [30]. PLL-treated cells show a cytoplasmic agglutination of nucleic acid and proteins, swollen mitochondria and binding of the polymer to selected areas on the cellular membrane [28]. Inhibition of MTT reduction by PLL following incubation with PLL is consistent with mitochondrial malfunction.

Taking all of the above factors into consideration, the MTT test was found to be as sensitive as the other tests previously reported to be useful in evaluating general cytotoxicity. Table V shows that the $IC_{50}$-values obtained here are in good agreement with those reported previously (various methods) for PLL exposed to a variety of cell types. The good correlation observed here for $IC_{90}$-values obtained using different viability assays shows that, even at low polymer concentrations, the MTT assay may be useful as a preliminary indicator and is still consistent with the other, more established, methods. The MTT assay would seem the logical choice as a method of preliminary screening of polymer toxicity. The method is simple, provides reproducible results and, unlike many other methods, does not use radioisotopes. All assays can be carried out using a fully automated microtitre plate system, economical with polymers of limited availability. We would emphasize that it is not suggested that the MTT test should replace the other viability-testing methods, but it is a useful test to precede and/or complement experiments using the other assays. After all, since the biological basis of each viability test is quite different and, as seen here, different cells may not respond in the same way, any rigorous

TABLE IV Cytotoxicity of soluble polymers measured using the MTT assay and $HepG_2$ cells in the absence of serum proteins*

| Concentration (mg ml$^{-1}$) | Viability (% untreated control) | | | | |
|---|---|---|---|---|---|
| | PLL† | PGA | PVP | DEX | PEG |
| 1 | 1.3 ± 1.6 | 110.9 ± 17.6 | 112.6 ± 17.0 | 106.5 ± 14.9 | 115.5 ± 16.6 |
| $10^{-1}$ | 0.7 ± 1.5 | 92.9 ± 12.4 | 99.1 ± 14.3 | 101.6 ± 17.6 | 98.6 ± 19.4 |
| $10^{-2}$ | 76.8 ± 9.5 | 92.1 ± 13.4 | 96.1 ± 13.1 | 100.2 ± 11.7 | 94.2 ± 11.8 |
| $10^{-3}$ | 107.1 ± 18.4 | 97.3 ± 17.8 | 97.3 ± 22.6 | 88.2 ± 11.2 | 91.8 ± 11.5 |
| $10^{-4}$ | 85.2 ± 13.5 | 99.7 ± 12.3 | 89.9 ± 12.8 | 90.7 ± 10.6 | 99.9 ± 18.4 |

*Incubation time 24 h, eight replicates per concentration, values shown are the mean ± s.e.m.
†Abbreviations shown in Table I.

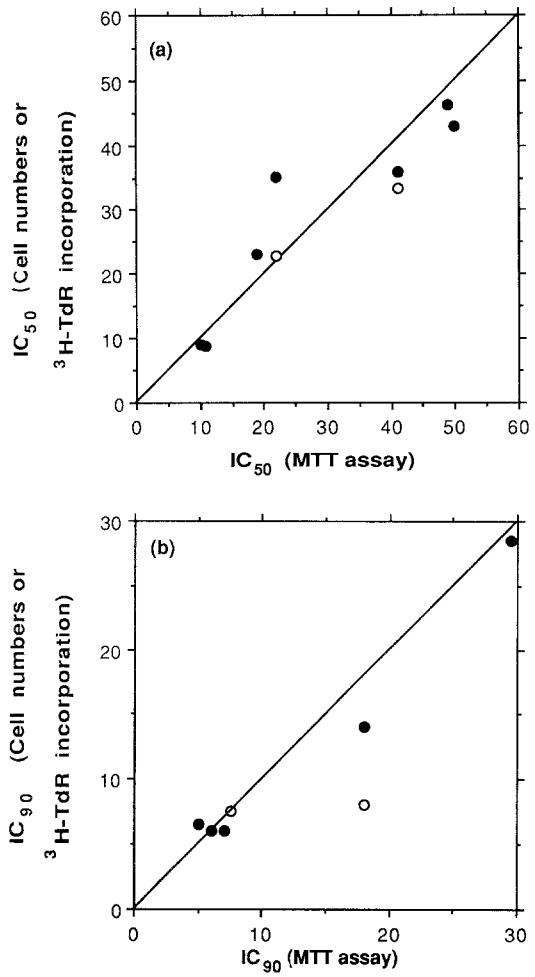


*Figure 5* Relationship between the $IC_{50}$- and $IC_{90}$-values of PLL (CCRF cells) when the MTT assay, [$^3$H]TdR and cell numbers were used as assay methods. (a) The relationship between the $IC_{50}$-values and (b) the $IC_{90}$-values. Each point represents the data obtained within a single experiment, cell numbers against MTT (●), [$^3$H]TdR incorporation against MTT (○).

examination would include a variety of screening methods.

To determine the overall biocompatibility of any new material it is necessary to carry out a wide variety of different tests. We have also developed rapid screening methods to characterize the haematoxicity

TABLE V Summary of $IC_{50}$-values estimated for PLL from past reports

| Cell line | Assay | Molecular weight* | $IC_{50}$ ($\mu g\,ml^{-1}$) | Reference |
|---|---|---|---|---|
| HeLa | Cell numbers | 35 000 | > 25 | 26 |
| HeLa | [$^3$H]TdR | 35 000 | > 10 | 26 |
| Huvec† | [$^3$H]Leu | 40 000 | > 32 | 30 |
| Huvec† | [$^3$H]Leu | 60 000 | < 48 | 30 |
| Huvec† | [$^3$H]Leu | 90 000 | < 72 | 30 |
| Huvec† | [$^3$H]Leu | 500 000 | > 20 | 30 |
| P388D$_1$‡ | LDH | 57 000 | < 50 | 33 |
| $HepG_2$ | MTT | 57 000 | 59.9 | § |
| $HepG_2$ | [$^3$H]TdR | 57 000 | 61.4 | § |
| CCRF | MTT | 57 000 | 23.9 | § |
| CCRF | [$^3$H]TdR | 57 000 | 35.9 | § |
| CCRF | Cell numbers | 57 000 | 28.7 | § |

*Mean average molecular weight.
†Human vascular endothelial cells.
‡Mouse mononuclear phagocytes derived from a methylcholanthene-induced lymphocyte neoplasm.
§Results have been taken from Table II.

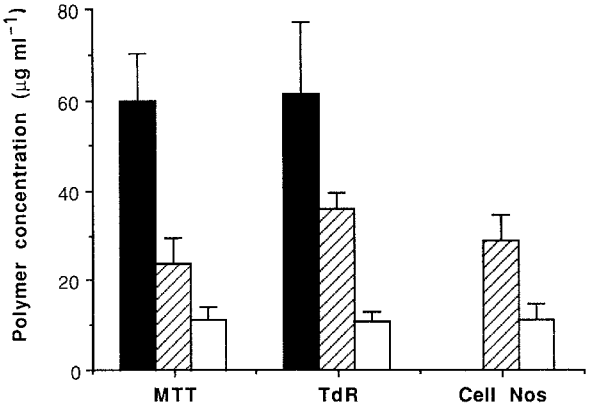


*Figure 6* $IC_{50}$- and $IC_{90}$-values of PLL against the $HepG_2$ (measured by the MTT and [$^3$H]TdR incorporation) or CCRF cell line (measured by the MTT, [$^3$H]TdR incorporation and cell numbers, respectively). (■) 50% Inhibition concentration of PLL ($HepG_2$), (▨) 50% inhibition concentration of PLL (CCRF) and (□) 10% inhibition concentration of PLL (CCRF).

(red blood cell lysis) of soluble polymers [31] and, using a series of reference compounds, determined relative *in vitro* toxicity (against a panel of cell lines) and their immunogenicity *in vivo* [32]. Our overall aim is to develop a portfolio of tests (including preliminary screens of toxicity, immunogenicity, carcinogenicity and material degradation) that can be calibrated with a series of reference polymers and later used to assess the potential of any new soluble polymeric material developed for biomedical use.

## 5. Conclusions

The MTT assay was compared (using $IC_{90}$- and $IC_{50}$-values) with standard methods for the assessment of viability of cultures, using a toxic polymer (PLL) and a number of non-toxic polymeric materials. The results obtained indicate that the MTT assay can serve as a reliable test for the primary evaluation of the cytotoxicity of soluble synthetic polymers, the advantages being that it is a simple, rapid and semi-automated assay. A number of cell-related factors were found to be important; cells should be used in the exponential phase of growth, the assay should be adjusted to give an optimum optical density for quantification of the MTT formazan precipitate and the pH of the medium should be carefully controlled. Although viability measured was independent of the assay method used (MTT, cell numbers, [$^3$H]TdR- or [$^3$H]Leu-incorporation), the results obtained were different for $HepG_2$ and CCRF cells, both in the presence or absence of serum proteins.

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