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Polyetheretherketone—cytotoxicity and mutagenicity in vitro

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Abstract

The results of the incubation of polyetheretherketone (PEEK) fibre material with seven different genotype variants of salmonella bacterium showed with and without an external metabolic activation system (S9) with no mutagenic or cytotoxic activity of the test material. In the so-called "plate incorporation test" in which the PEEK raw material is finely cut and applied direct to the agar plate without the addition of solvent there was, as expected, no evidence of cytotoxic or mutagenic effects.

In the HPRT test there was a significant increase in the number of mutants per dish, both after addition of *N*-acetylaminofluorene and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (with and without an external metabolic activation system = ±S9), but not after treatment of the cells with PEEK-DMSO-eluate. This means that the PEEK material under study did not release any substances that cause V79 cells to mutate. The investigation of the toxic reaction on the material under study revealed that the number of surviving colonies per 10^5 surviving cells lay within the range of or below the solvent control even in the presence of high PEEK concentrations (5.0 μg/ml). Therefore, in summary, the study produced no evidence of cell damage caused by PEEK. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Polyetheretherketone; Cytotoxicity; Mutagenicity; Ames test; HPRT test

1. Introduction

Recognition of an implant material as biocompatible nowadays depends on a large number of factors, such as:

- Absence of cytotoxicity, mutagenicity, carcinogenicity
- Exclusion of allergenic properties
- Physical-chemical and biological "inertia"
- Stability in its biological environment

Therefore, before new materials are approved for medical use mutagenesis systems to exclude cytotoxic, mutagenic or carcinogenic properties are applied worldwide. Although indispensable within the framework of in-vitro evaluation, these screening procedures are usually very work-intensive and time-consuming. They must be carried out for the raw material as well as the manufactured implant in order to exclude the possibility that the properties of the material are influenced by the industrial manufacturing process.

If such materials are intended for use in medicine, the combination of two different mutagenesis studies (e.g. bacteria and mammalian cells) continues to be necessary for safety reasons. To ensure the clarity of the results it is recommended that study conditions be based on an internationally accepted standard.

Nowadays, however, there are more than one hundred different testing methods for collecting evidence of carcinogenic and mutagenic activity. Many of them are based on the principle that genotoxicity or mutagenicity serves as an indicator for the carcinogenic potential of a substance [1]. It is neither necessary nor practicable to employ all known methods in one test series [2], but results from one model experiment only are not conclusive enough. Based on the recommendations of the WHO, ISO (ISO 10993) and the ASTM [3], therefore, two different representative test methods for

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the detection of cytotoxic and mutagenic effects of the implant material polyetheretherketone (PEEK) were applied and each test series was repeated at least once:

Mutagenesis and toxicity in bacteria: *Salmonella typhimurium* (Ames Test)

Mutagenesis in mammalian cells: Chinese hamster fibroblasts (V79)

2. Materials and methods

2.1. Polyetheretherketone (PEEK)

Polyetheretherketone (PEEK 381G, CAS 57947-42-9) is an aromatic, semi-crystalline linear polymer with the repeat unit oxy-1.4-phenyl-oxy1.4-phenyl-carbonyl-1-4-phenyl, which can be synthesised from hydroquinone and 4.4-difluorbenzophenon (Fig. 1).

This thermoplastic has high glass transition (143°C) and melting temperatures (334°C). Pure PEEK polymer 381G retains its shape stability up to 152°C, the fibre-reinforced compounds up to 315°C (ISO R75). The temperature during prolonged use and heat conductivity at about 260°C is 0.25 W/m/°C, respectively. Due to its stable chemical structure the polymer is largely resistant to water, high-pressure steam and ionizing radiation (changes in its bending properties do not occur until gamma ray doses of >109 rad). This means that the material can be repeatedly sterilised by all the usual methods (heat sterilisation: 170–180°C, moist heat sterilisation: 200°C/1 bar, gamma sterilisation) without change in its physical properties. The manufacturer (VICTREX plc) guarantees inherent purity of the material with minute quantities of extractable ions or gas emission.

The material can be manufactured using conventional thermoplastic machines without the need for a tempering process. PEEK is mainly processed by extrusion and injection moulding. Furthermore, it is used as a matrix for glass or carbon fibre reinforced composite materials where an extremely close bond between matrix and fibre reinforcement results. An important advantage for its use in medicine is that PEEK can be processed without additives. Due to its good resistance to commercially available chemicals, PEEK can be dissolved practically only in concentrated sulphuric acid [4–7].

Pure PEEK 381G polymer is available in the form of grey granules of medium melting viscosity and is especially suitable for the manufacture of foil and monofilament. Its density, measured according to ISO R1183, is about 1.32 g/cm^3 in the crystalline state, 1.26 g/cm^3 in the amorphous state and its typical crystallinity is about 35%. According to ISO R262A the material absorbs only 0.5% water at 23°C over a period of 24 h and also in equilibrium. Its tensile strength at a constant test speed of 50 mm/min corresponds to 97 N/mm^2 (23°C) and 12 N/mm^2 (250°C; ISO R527). At the same test speed the limit of elasticity is about 5% and the strain until failure about >60% (23°C, ISO R527). The bending E-modulus is given according to ISO R178 as 4.1 or 4.0 GPa (23°C/120°C) and 0.3 GPa (250°C), the flexional resistance as 170 MPa (23°C) or 100 MPa (120°C) and 13 MPa (250°C). According to ASTM standard D3846 the transverse resistance and rigidity modulus—measured at 23°C—are 53 MPa and 1.3 GPa, respectively. PEEK absorbs only small quantities of protein structures from biological media, other interactions with biological substances are unknown. PEEK therefore fulfils the US Pharmakopöe (USP) requirements, Class VI. In the medical field, the material was at first mainly used to manufacture components for medical devices (e.g. parts for kidney dialysis machines, GAMBRO/Sweden), analysis equipment (fluid contact chromatography systems) and instruments. In the meantime it is available under the name PEEK Optima^{TM} LT Polymer specifically for medical purposes, for instance as an implant material [6,7].

Although linear polymers such as PEEK are in principle more susceptible to degradation than plastics with branched molecule chains, it is specifically the hydrophobic properties of the material and the correspondingly poor wettability by water-soluble substances which suggest that PEEK is very stable in the presence of enzymatically controlled decomposition processes, as these are usually strictly polar due to functional groups such as –COOH, –OH, and –NH_2 and therefore hydrophile. A further property of PEEK which is an advantage for its use as a prosthetic material is its weldability.

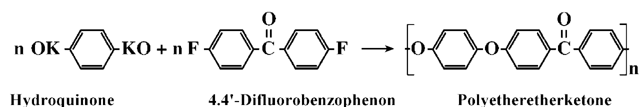

Fig. 1. Synthesis and structural formula: polyetheretherketone.

2.2. Ames test

The method developed by B. Ames, J. McCann and E: Yamasaki was published in 1975 [8,9]. The test substance is incubated with special genotype variants of the bacterium *Salmonella typhimurium* which carry mutations in several genes. Seven strains were used (TA 97, TA 98, TA 100, TA 102, TA 1535, TA 1537, TA 1538)[1] with a known mutational pattern in histidine-operone, so they depend on exogenic histidine and in contrast to the so-called "wild type" are not able to

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grow on histidine-free agar. By contact with a mutagenic test substance the mutations of the histidine genes can be reverted, so that the bacteria grow again as revertants on histidine-free agar independently of exogenic histidine supply and can be counted in the form of colonies. By addition of rat liver homogenate (supernatant 9000 *g* acceleration) activation of mutagens is possible.

2.2.1. Experimental method

The bacteria are taken from the strain culture stored at −80°C under sterile conditions and are injected into about 20 ml of a nutrient solution (25 g nutrient broth from OXOID Ltd. ad 1000 ml aqua dest.). This solution is then shaken in an incubator for 7 h at 37°C (55 rpm).

Two different sorts of agar are needed:

(1) Bottom agar, consisting of 1.5% oxoid-bacto-agar in Vogel–Bonner medium E (per litre H_2O : 0.2 g $MgSO_4$ 7 H_2O, 2 g citric acid, 10 g $KH_4(PO_4)_4$, 3.5 g NaH_2 $(PO)_4 4H_2O$) with 2% glucose. 30 ml of the autoclaved agar is poured into a sterile Petri dish ($100 \times 15\,mm^2$). After cooling, the agar dishes can be kept for quite a long period of time at 4°C.
(2) Top agar, consisting of 0.6% oxoid-agar and 0.6% sodium chloride. 10 ml of histidine-biotin solution (96 mg L-histidine × HCl and 123, 6 mg biotin/l) is added to each measure of 100 ml of top agar.

The L-histidine promotes expression ("mutagenic hit") of the revertants, but is not sufficient to allow the histidine-dependent bacteria to mature into a colony. The addition of biotin is necessary because the "uvrB" deletion reaches into the neighbouring biotin gene. The plate incorporation test is carried out by covering the bottom agar with a layer of 2 ml of top agar, the bacteria, the test substance and, if necessary, S9-Mix (10 µl of a rat liver homogenate filtered at 9000 *g* and 90 µl of a 0.2 mol phosphate buffer containing 8 µmol magnesium chloride, 33 µmol potassium chloride, 5 µmol glucose-6-phosphate and 4 µmol NADP).

The test series described here were carried out as a liquid preincubation test [10], in which a mixture of 100 µl of bacteria suspension and 100 µl of phosphate buffer or S9-Mix and test material (either 20 µl of Dimetylsulfoxide (DMSO)-extract or finely ground raw material) is preincubated for 30 min at 37°C under constant shaking (120 rpm), then mixed with top agar and plated.

The PEEK test material was prepared as follows:

1 g of very finely cut PEEK was shaken in 10 ml of ethanol or chloroform at 37°C for 24 h. The supernatant was decanted and filtered, then concentrated in a rotary evaporator and dissolved in 2 ml of DMSO. The solvents were treated in the same way. To test the raw material, 50 mg of very finely cut PEEK fibre material were mixed with 350 µl of bacterial suspension and 350 µl of phosphate buffer or enzyme preparation (S9-Mix), then pre-incubated at 37°C and shaken constantly (120 rpm), finally in portions of 200 µl mixed with 2 ml of top agar and plated (Plate incorporation test).

In addition to the test samples every test series included negative controls in different doses to monitor spontaneous revertants as well as solvent controls. At the same time positive controls were carried out in which the corresponding salmonella strain was tested with spontaneously active mutagens (2 nitrofluorene or sodium azide) and mutagens requiring metabolic activation (2 aminoanthracene) by addition of S9 (enzyme control).

The colonies were counted automatically (Accucount, ARTEC Syst. Corp.). Parallel to the mutagenesis test, toxicity tests were carried out following the same procedure. The bacterial cultures grown overnight were diluted with 0.9% NaCl or buffer until an easily countable quantity of bacteria was present in the Petri dishes. This time nutrient agar was used as bottom agar to enable growth of the histidine-dependent mutants. Cytotoxic effects of the test substance on other strains were only registered for values which were more than twice as high as the negative control (see below).

The test results were evaluated after an incubation period of two days. The counted colonies were compared with the negative and positive controls, with the solvent control, and then the toxicity determined. The cytotoxicity and mutagenicity of PEEK were investigated both in standard (30 min incubation) and long-term (up to 4 h pre-incubation) evaluations.

2.3. Mutagenesis in mammalian cells: HPRT test

HPRT (hypoxanthine-guanine-phosphoribosyl-transferase) is an enzyme whose gene is located on the X chromosome. The enzyme catalyses the conversion of the purines hypoxanthine and guanine into nucleosides and in combination with phosphoribosyl-pyrophosphate into nucleoside-5′-monophosphates. Purine analogues such as 6-mercaptopurine, 6thioguanine or 8-azaguanine are also metabolised by HPRT, but these substances have a cytotoxic effect and the nucleosides formed from them cause cell death.

In cell culture spontaneous mutation occurs involving the HPRT gene among others. As a result these cells cannot form the enzyme. Therefore, these mutants are able to grow in a medium that contains purine analogues, while cells with normal HPRT gene die in it. The mutation rate is increased by mutagenic substances through different mechanisms (e.g. base pair substitution, chromosome aberrations) so that the number of surviving cells in a corresponding selection medium represents a measure for the mutagenic efficiency of the test substance (Fig. 2).

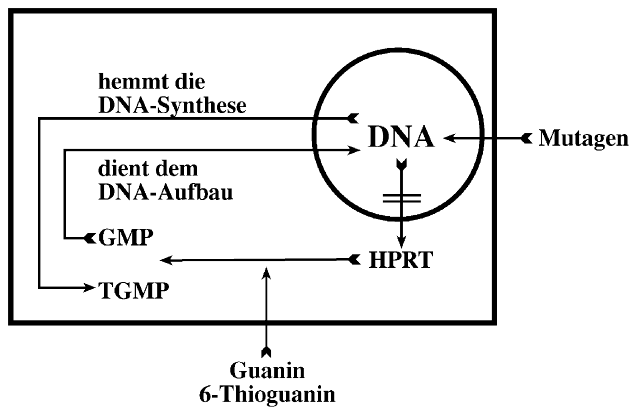

Fig. 2. Principle of the HPRT mutation test.

2.3.1. Cells

The V79 cell-line of the Chinese hamster was employed in this part of the study. The cells were kindly provided by Dr. E.H.Y. Chu.[2] The V79 cells were kept in a growth medium (Dulbecco's Minimum Essential Medium = DMEM) with 8% dimethylsulfoxyd (DMSO) at −80°C or in liquid nitrogen. The targeted cell density was about 10^6/ml. The cell suspension was thawed in a water bath at 37°C and transferred to a cell culture flask made of plastic (75 cm^2) with 10 ml of growth medium. After about 4–5 h the cells had adhered to the floor of the flask. The medium was poured off and replaced by 20 ml of new medium. The cells were incubated in an incubator at 37°C, 5% CO_2 and 90% humidity.

3. Results

3.1. Ames test

The test substance is considered to be mutagenic when the number of counted colonies exceeds the number of colonies in the negative controls by at least double, and a relationship between dose and response can be observed.

3.1.1. Mutagenesis test

To give a clear presentation of the results these are summarised in the following block diagrams (Fig. 3a–n).

$$Q(+\mathrm{S9}) = \frac{\text{Rev sample } (+\mathrm{S9})}{\text{Rev control } (-\mathrm{S9})}.$$

$$Q(-\mathrm{S9}) = \frac{\text{Rev sample } (-\mathrm{S9})}{\text{Rev control } (+\mathrm{S9})}.$$

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3.1.2. Toxicity test

As representative for all bacteria strains on which the mutagenesis test was carried out the individual results of the toxicity test for the TA 98 strain with and without metabolic activation (S9) are presented as block diagrams (o + p; Fig. 4).

The columns C, NF, SA, MC, AAC, EC and CC represent control groups. C stands for negative control and gives the number of spontaneous revertants for the mutagenesis test, while in the toxicity test C stands for the normal survival rate. NF, SA, MC and AAC are positive controls whereby NF represents the "frame-shift" strains TA 97, TA 98 and TA 1538 without an external metabolic activation system (−S9), SA the reaction of the "base pair" substituted strains TA 100 and TA 1535 (−S9), MC only the reaction of the TA 102 (−S9) strain and AAC the reaction of all strains except TA 102 with and without activator (±S9). D stands for the reaction of the TA 102 strain (+S9) to danthron (Table 1).

3.2. HPRT test

The results of the HPRT Test are summarised in the Tables 2 and 3.

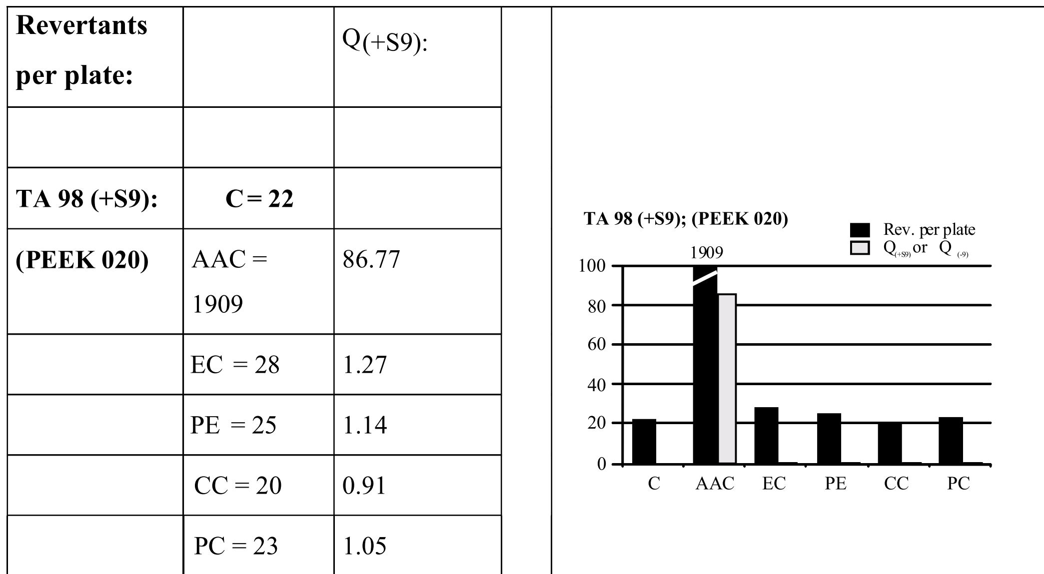
4. Discussion

To reduce the number of biomechanical studies, and above all unnecessary animal trials, evaluation of new biomaterials should nowadays begin with in vitro cytotoxicity and mutagenicity tests. This applies for the development of both temporary and permanent implants and prostheses, and for permanent implants particularly under the aspect that carcinogenic potential is often the consequence of chronic exposure to minute concentrations.

Although all known assays can yield false-positive and false-negative results, experience shows that the combination of two different test methods is a reliable parameter for determining carcinogens which are a risk to human health. This is why in this study we carried out two independent cytotoxicity and mutagenicity test series, one employing the bacterium Salmonella typhimurium (Ames Test) and the other employing mammalian cells (Chinese hamster fibroblasts, V-79).

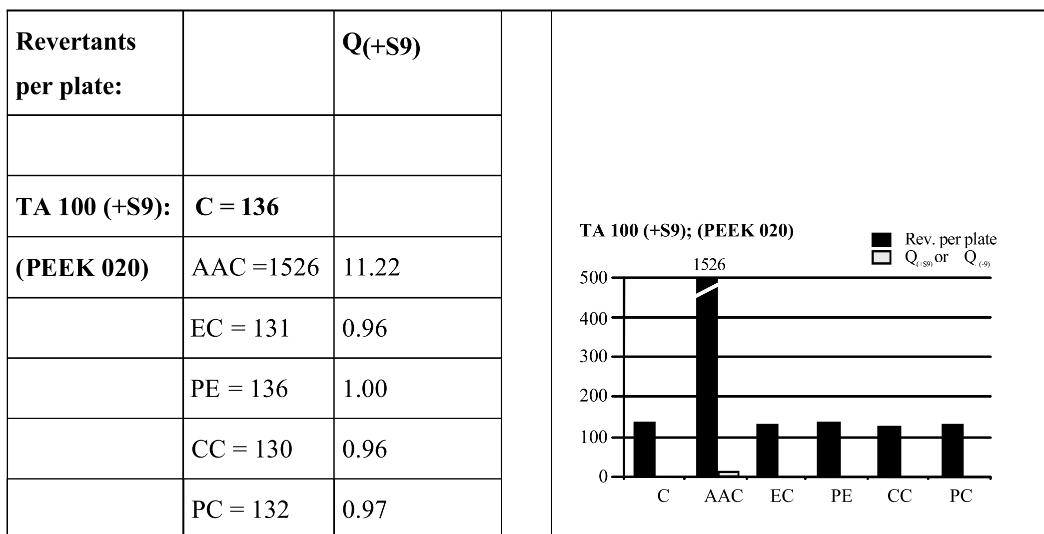
Extraction time for the test substance ranges between 1–3 and 72 h in literature (147). We chose 24 h which corresponds to the standard extraction time recommended by the majority of authors [11,8,10,9]. The incubation temperature of 37°C approximately corresponds to human body temperature, which is the temperature at which the polymer is intended for use. We dispensed with further tests at higher or lower

(a)



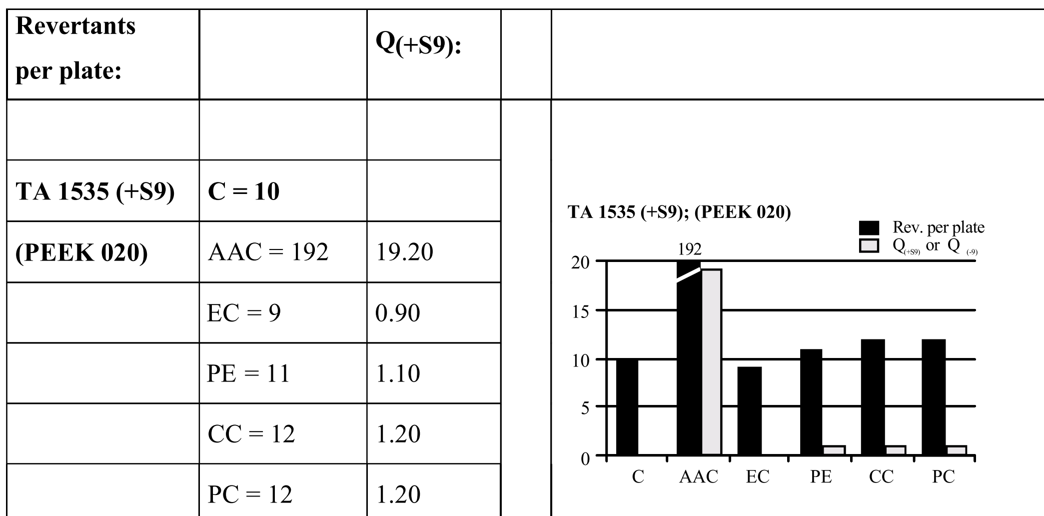
Revertants per plate:		Q(+S9):
TA 98 (+S9):	**C = 22**	
(PEEK 020)	AAC = 1909	86.77
	EC = 28	1.27
	PE = 25	1.14
	CC = 20	0.91
	PC = 23	1.05

(b)



Revertants per plate:		Q(+S9)
TA 100 (+S9):	**C = 136**	
(PEEK 020)	AAC =1526	11.22
	EC = 131	0.96
	PE = 136	1.00
	CC = 130	0.96
	PC = 132	0.97

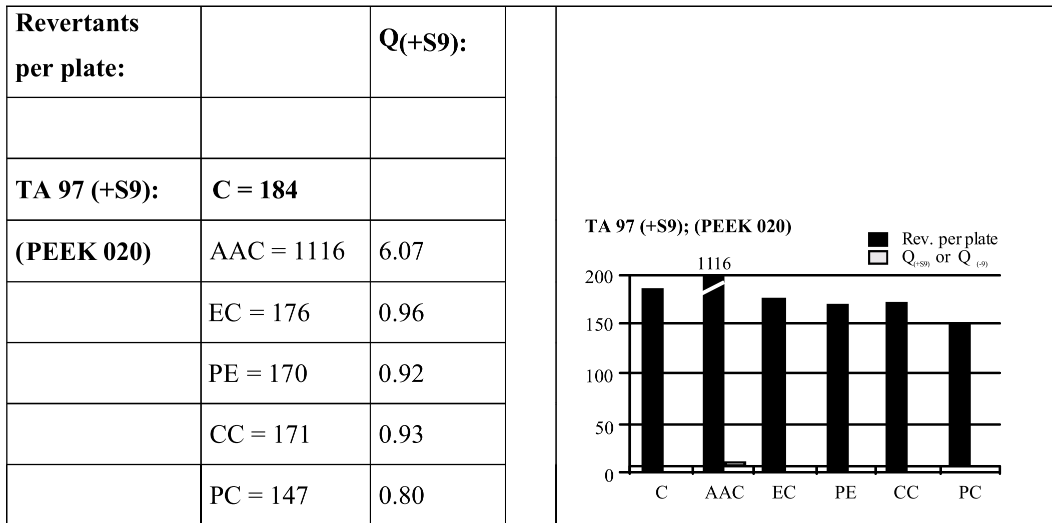
(c)



Revertants per plate:		Q(+S9):
TA 1535 (+S9)	**C = 10**	
(PEEK 020)	AAC = 192	19.20
	EC = 9	0.90
	PE = 11	1.10
	CC = 12	1.20
	PC = 12	1.20

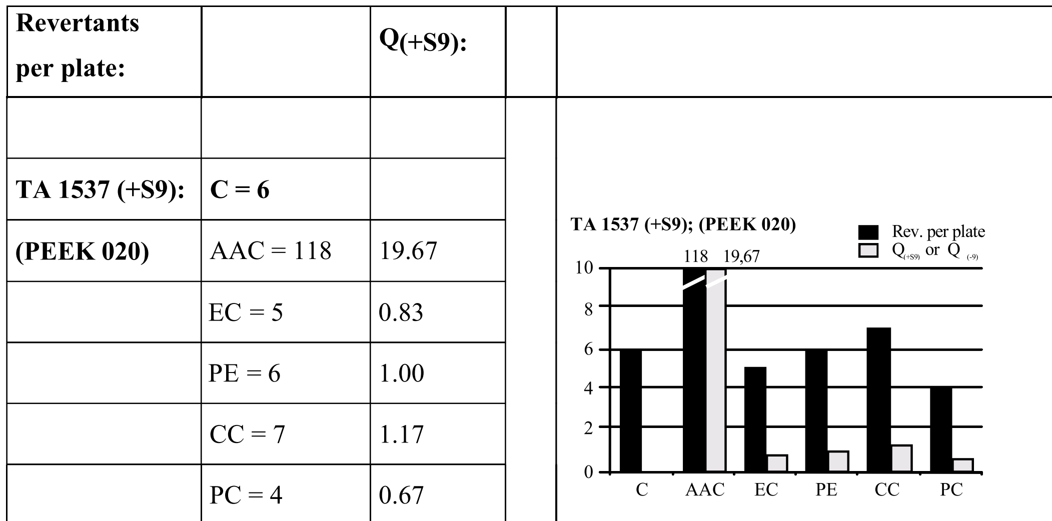
Fig. 3. (a–n) Mutagenesis test/block diagrams.

(d)



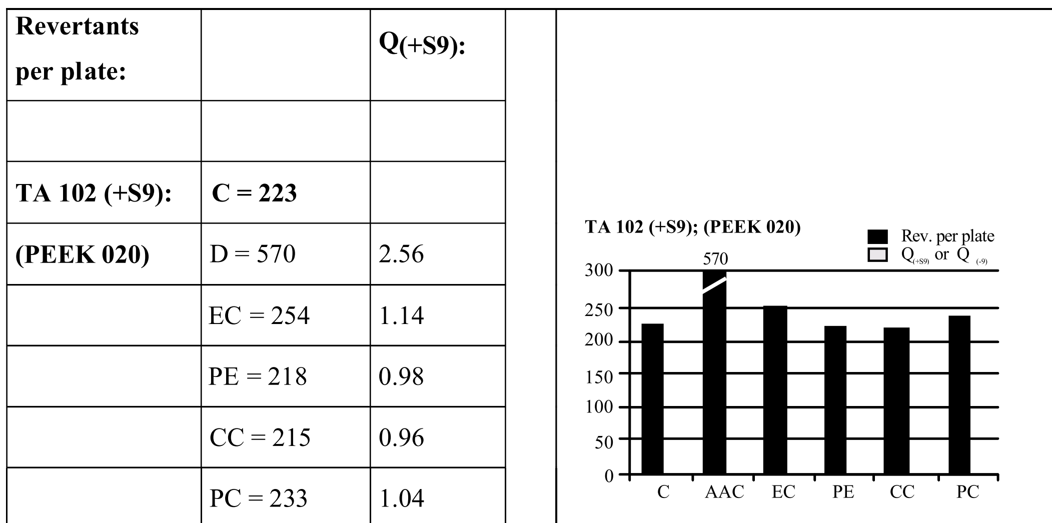
Revertants per plate:		Q(+S9):
TA 97 (+S9):	**C = 184**	
(PEEK 020)	AAC = 1116	6.07
	EC = 176	0.96
	PE = 170	0.92
	CC = 171	0.93
	PC = 147	0.80

(e)



Revertants per plate:		Q(+S9):
TA 1537 (+S9):	**C = 6**	
(PEEK 020)	AAC = 118	19.67
	EC = 5	0.83
	PE = 6	1.00
	CC = 7	1.17
	PC = 4	0.67

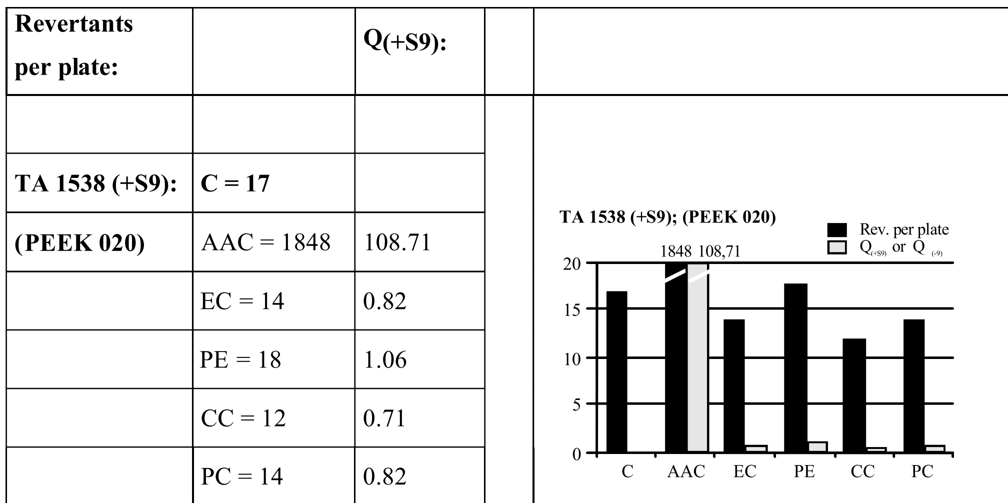
(f)



Revertants per plate:		Q(+S9):
TA 102 (+S9):	**C = 223**	
(PEEK 020)	D = 570	2.56
	EC = 254	1.14
	PE = 218	0.98
	CC = 215	0.96
	PC = 233	1.04

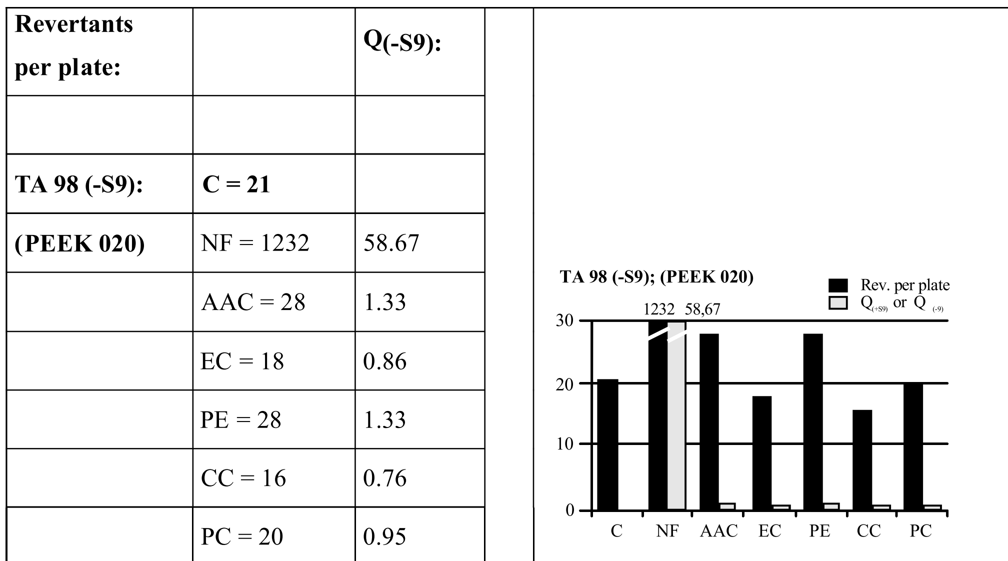
Fig. 3. (*continued*).

(g)



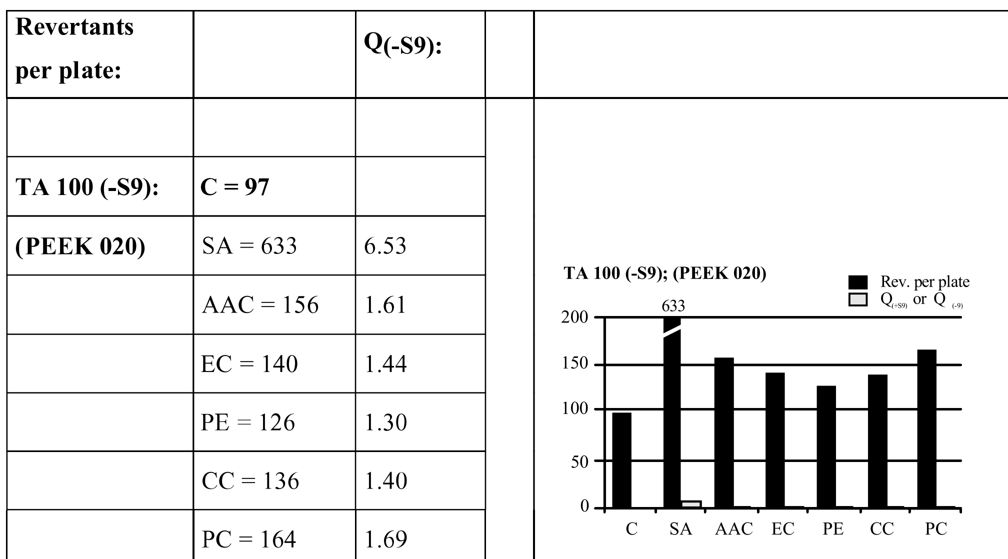
Revertants per plate:		Q(+S9):
TA 1538 (+S9):	**C = 17**	
(PEEK 020)	AAC = 1848	108.71
	EC = 14	0.82
	PE = 18	1.06
	CC = 12	0.71
	PC = 14	0.82

(h)



Revertants per plate:		Q(-S9):
TA 98 (-S9):	**C = 21**	
(PEEK 020)	NF = 1232	58.67
	AAC = 28	1.33
	EC = 18	0.86
	PE = 28	1.33
	CC = 16	0.76
	PC = 20	0.95

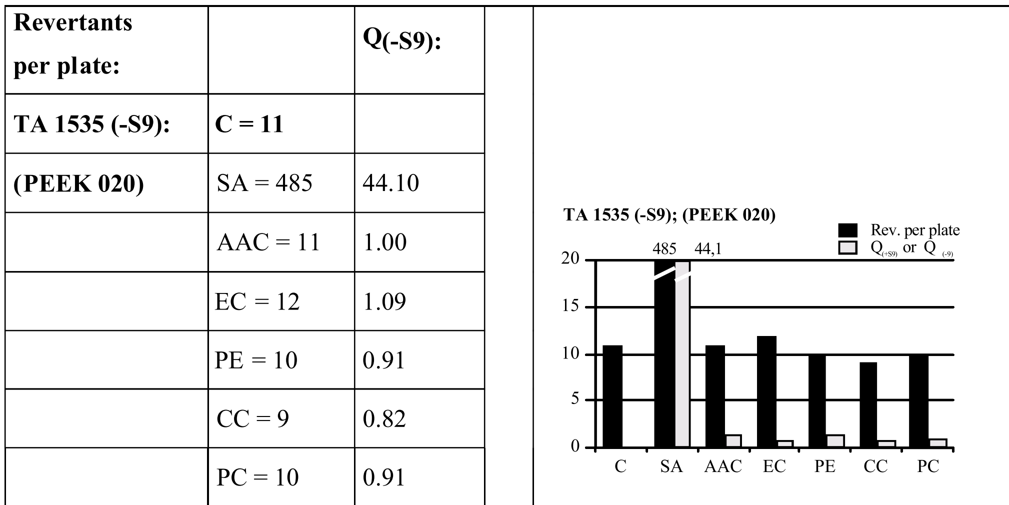
(i)



Revertants per plate:		Q(-S9):
TA 100 (-S9):	**C = 97**	
(PEEK 020)	SA = 633	6.53
	AAC = 156	1.61
	EC = 140	1.44
	PE = 126	1.30
	CC = 136	1.40
	PC = 164	1.69

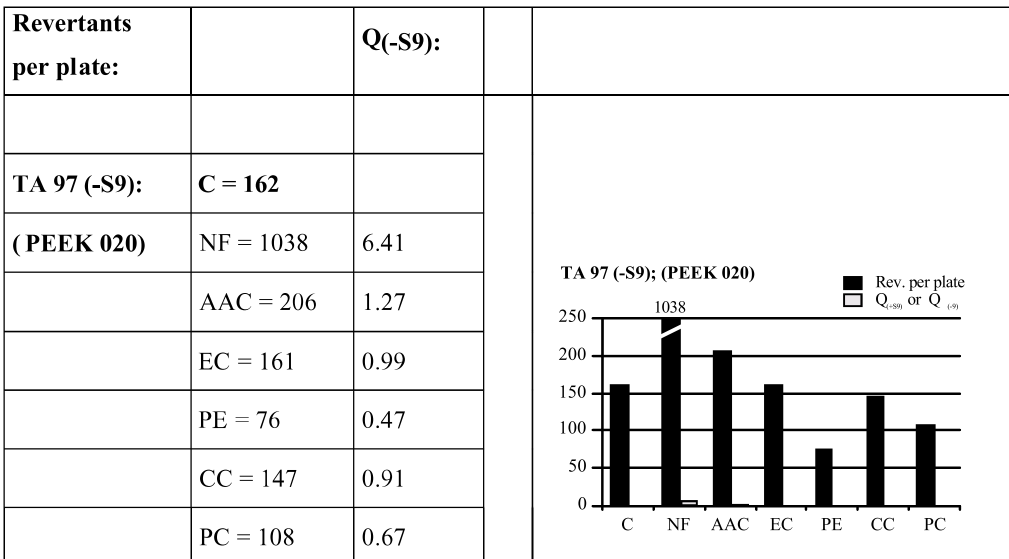
Fig. 3. (*continued*).

(j)



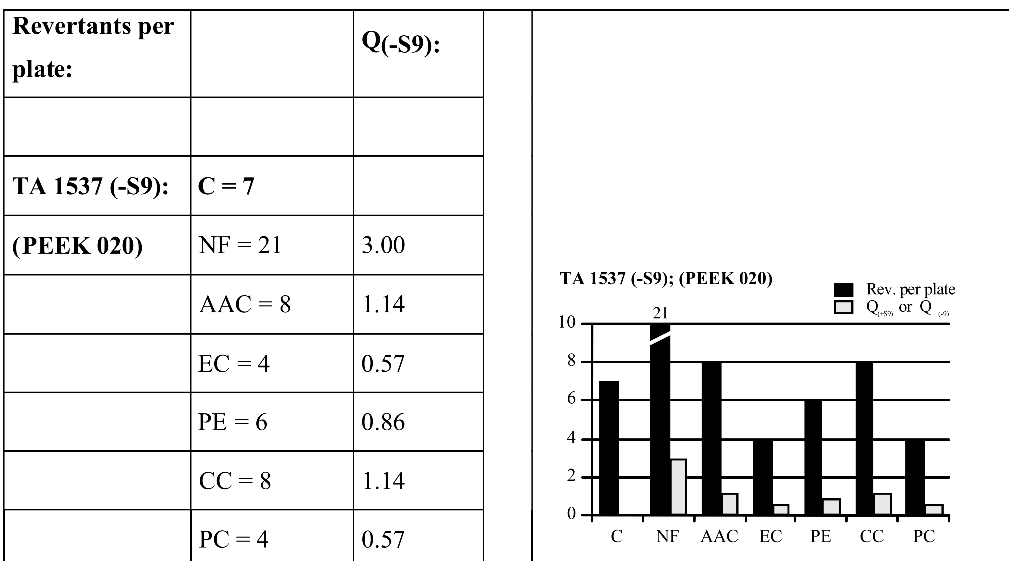
Revertants per plate:		Q(-S9):
TA 1535 (-S9):	**C = 11**	
(PEEK 020)	SA = 485	44.10
	AAC = 11	1.00
	EC = 12	1.09
	PE = 10	0.91
	CC = 9	0.82
	PC = 10	0.91

(k)



Revertants per plate:		Q(-S9):
TA 97 (-S9):	**C = 162**	
(PEEK 020)	NF = 1038	6.41
	AAC = 206	1.27
	EC = 161	0.99
	PE = 76	0.47
	CC = 147	0.91
	PC = 108	0.67

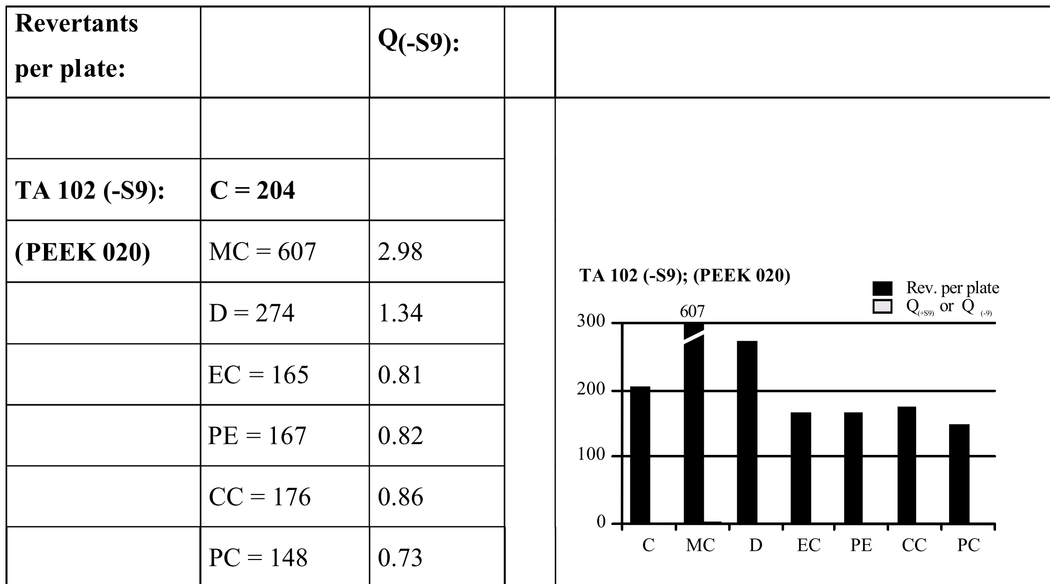
(l)



Revertants per plate:		Q(-S9):
TA 1537 (-S9):	**C = 7**	
(PEEK 020)	NF = 21	3.00
	AAC = 8	1.14
	EC = 4	0.57
	PE = 6	0.86
	CC = 8	1.14
	PC = 4	0.57

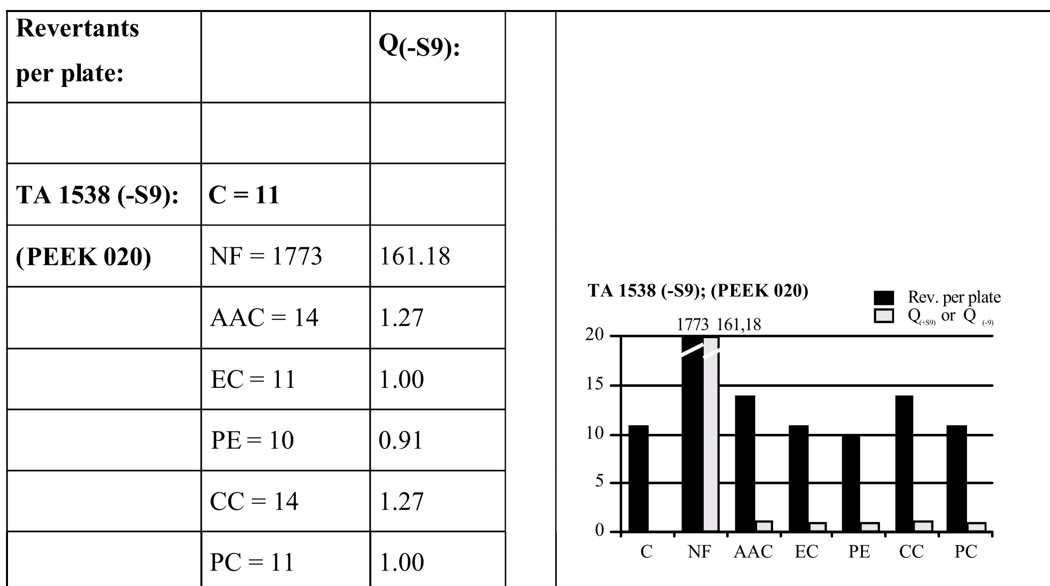
Fig. 3. (*continued*).

(m)



Revertants per plate:		Q(-S9):
TA 102 (-S9):	**C = 204**	
(PEEK 020)	MC = 607	2.98
	D = 274	1.34
	EC = 165	0.81
	PE = 167	0.82
	CC = 176	0.86
	PC = 148	0.73

(n)



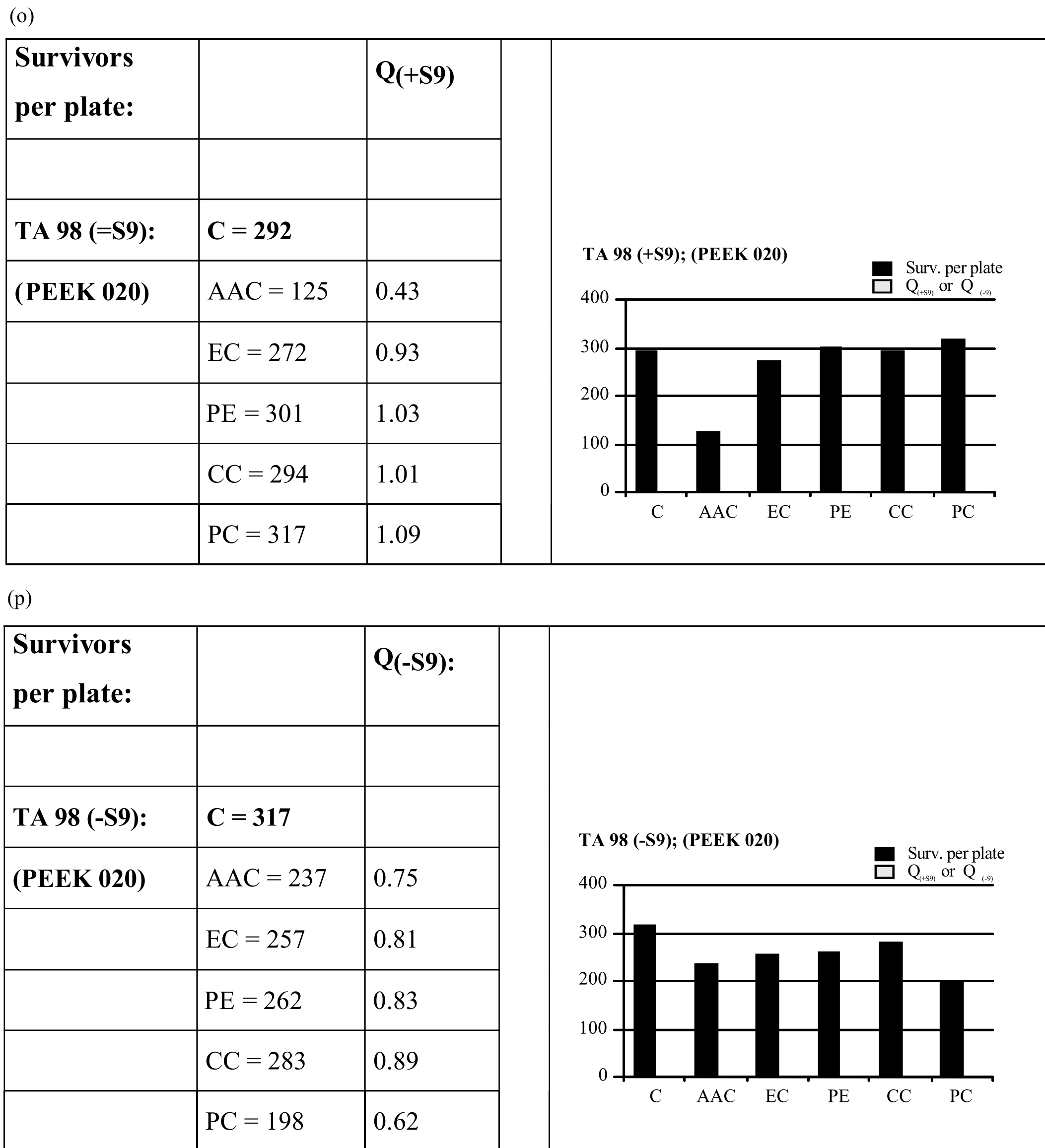
Revertants per plate:		Q(-S9):
TA 1538 (-S9):	**C = 11**	
(PEEK 020)	NF = 1773	161.18
	AAC = 14	1.27
	EC = 11	1.00
	PE = 10	0.91
	CC = 14	1.27
	PC = 11	1.00

Fig. 3. (*continued*).

temperatures in this screening series as PEEK is largely inert up to a continuous temperature of 260°C [6].

According to other studies in the literature detailing aspects of the biocompatibility of PEEK [12] our results show that neither PEEK braid nor its ethanol or chloroform extracts induce mutagenicity or cytotoxicity under the chosen study conditions. At the same time the importance of the washing procedure (methylenchloride and methanol) for industrially manufactured prostheses before use in animal trials becomes very clear, as particularly the ethanol extracts of PEEK raw material (= "unwashed" PEEK from the roll) both with and without an external metabolic activation system exhibited some mutagenic activity in preliminary experiments, above all in strains 1537 and 1538. There was no further evidence of this effect in repeated control tests with the (washed) implant. To exclude the possibility that results might be influenced by fibre length great care was taken during the cutting of PEEK samples to ensure that the particles were of identical size.

To summarise, in vitro testing with different *Salmonella typhimurium* strains produced no evidence of mutagenic or cytotoxic activity of PEEK on the human organism and therefore justified biocompatibility testing


(o)

Survivors per plate:		**Q(+S9)**
TA 98 (=S9):	**C = 292**	
(PEEK 020)	AAC = 125	0.43
	EC = 272	0.93
	PE = 301	1.03
	CC = 294	1.01
	PC = 317	1.09

(p)

Survivors per plate:		**Q(-S9):**
TA 98 (-S9):	**C = 317**	
(PEEK 020)	AAC = 237	0.75
	EC = 257	0.81
	PE = 262	0.83
	CC = 283	0.89
	PC = 198	0.62

Fig. 4. (o–p) Toxicity test/block diagrams.

Table 1
Legend to Ames Test

C	Control	Negative control	
Nf	2-nitrofluorene	Positive control	−S9
Sa	Sodium acid	Positive control	−S9
Mc	Mitomycin	Positive control	−S9
Aac	2-aminoanthracene	Positive control	+S9
D	Danthron	Positive control	+S9
Ec	Ethanol	Solvent control	
Cc	Chloroform	Solvent control	
Pe	PEEK ethanol extract		
Pc	PEEK chloroform extract		
Pg	PEEK raw material		

on animals. These results correlated with studies on carbon-fibre reinforced PEEK compounds where the investigation of lactatedehydrogenase (LDH) activity in cell cultures of mouse fibroblasts (L929) as proliferation markers over 120 h showed that carbon-fibre reinforced PEEK, both on direct contact as well as in extraction tests, has a very good biocompatibility with no indication of cytotoxic effects [13].

Table 2
V-79 Test: PEEK with metabolic activation (+S9)

Polyetheretherketone[a] +S9				
Treatment	Concen. (μg/ml)	CFE (%)	Mutants/dish	Mut./10^5 surv. cells
1. Control	—	25.0	3.4+/−2.8	2.7
2. AAF[b]	20	15.7	22.2+/−1.9	28.2
3. PEEK	0.5	28.5	5.4+/−2.3	3.8
4. PEEK	1.5	22.5	5.4+/−1.8	4.8
5. PEEK	5.0	24.0	3.2+/−1.8	2.7

[a] 800 mg polyetheretherketone incorporating 8 ml ethanol, incubated for 24 h at 37°C under const. shaking, supernatant pipetted off, concentrated in a rotatory evaporator and dissolved in 1 ml DMSO.
[b] *N*-acetylaminofluorene dissolved in medium with metabolic activation = positive control substance.

Table 3
V-79 Test: PEEK without metabolic activation (−S9)

Polyetheretherketone −S9				
Treatment	Concen. (μg/ml)	CFE (%)	Mutants/dish	Mut./10^5 surv. cells
1. Control	—	19.1	4.0+/−2.5	4.2
2. MNNG[a]	1.0	22.0	63.0+/−3.7	47.7
3. PEEK	0.5	26.4	3.2+/−0.8	2.4
4. PEEK	1.5	26.1	6.2+/−5.1	3.7
5. PEEK	5.0	33.6	4.0+/−1.7	3.6

[a] *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, dissolved in medium without metabolic activation = positive control substance.

Studies on SV40 rat osteoblasts and 3T3 mouse fibroblasts in direct contact with the material revealed the same results both for pure and carbon-fibre reinforced PEEK: after 48 h incubation there were no effects on the morphology of the osteoblasts nor was there any evidence of a negative influence on the 3T3 proliferation rate or cytotoxic effects on the osteoblasts in the MTT assay. On the contrary, there was even evidence of stimulation of the osteoblast protein content which has resulted in discussion that PEEK might have a favourable effect on bone growth (osteointegration) (106). Further proliferation studies with direct material contact were carried out with rat tail and UMR 106.1 osteoblast cultures. The cell proliferation rate—measured by incorporation of 3 H tymidin corresponded to the values of the negative control [14].

Analyses of morphology and proliferation rate of the human bone marrow cell line MG63 after incubation with a PEEK glass fibre revealed no negative effect on osteoblast morphology or cell density. Again, there was evidence of stimulation of cellular synthesis by PEEK due to an increased osteocalcin level and greater Alkaline phosphatase (AP) activity [15].

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