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Synthesis and biological evaluation of PMMA/MMT nanocomposite as denture base material

Junping Zheng · Qiang Su · Chen Wang · Gang Cheng · Ran Zhu · Jin Shi · Kangde Yao

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Abstract Inorganic-polymer nanocomposites are of significant interest for emerging materials due to their improved properties and unique combination of properties. Poly (methylmethacrylate) (PMMA)/montmorillonite (MMT) nanocomposites were prepared by in situ suspension polymerization with dodecylamine used as MMT-modifier. X-ray diffraction (XRD) and transmission electron microscopy (TEM) were used to characterize the structures of the nanocomposites. Cytotoxicity test, hemolysis test, acute systemic toxicity test, oral mucous membrane irritation test, guinea-pig maximization test and mouse bone-marrow micronucleus test were used to evaluate the biocompatibility of PMMA/MMT nanocomposites. The results indicated that an exfoliated nanocomposite was achieved, and the resulting nanocomposites exhibited excellent biocompatibility as denture base material and had potential application in dental materials.

1 Introduction

The development of strategies for the synthesis of polymer/montmorillonite (MMT) nanocomposites is rapidly growing mainly because of enhanced properties emerging from such kind of materials [1, 2]. Polymer/MMT nanocomposites have been reported a lot these years. With only a low content of MMT, the strength, Young's modulus, and solvent resistance of the composites can be greatly improved [3]. Among these nanocomposites, Poly (methylmethacrylate) (PMMA) has been widely used due to several desirable properties including exceptional mechanical–thermal and dimensional stability, process ability, and also has been applied to various prosthetic replacement operations such as bone substitution and dental materials [4]. PMMA/MMT nanocomposites have been reported to be prepared by bulk polymerization, solution polymerization, emulsion polymerization, suspension polymerization and sol–gel method [5–7]. PMMA and PMMA-based composites prepared by suspension polymerization have always been applied in dentures [8], surgical bone cements [9], drug delivery systems [10] and so on. After Huang and Brittain [11] firstly reported the synthesis of PMMA/MMT nanocomposites via suspension polymerization, many researchers paid their attention on the research of PMMA/MMT denture base material. For instance, Salahuddin [8] incorporated an organophilic MMT to solve the problem of polymerization shrinkage in PMMA as dentures, the results indicated that polymer–clay composites exhibit significant increase in thermal stability with very small amount of inorganic content. Kim [12] prepared PMMA beads with MMT via suspension polymerization method for applying acrylic bone cements, and the effects of preparation conditions on the size and shape of the polymer beads were discussed. Mohammad et al. [13] prepared PMMA-grafted nanoclay used as novel filler for dental adhesives. Discacciati [14] has reported that the possibility of producing photopolymerized dental resins containing exfoliated nanolayers can potentially be useful in controlling important properties of dental materials such as resistance to attrition, moisture absorption, polymerization shrinkage, coefficient of thermal expansion, among others. However, there are few reports about

J. Zheng (✉) · Q. Su · G. Cheng · R. Zhu · K. Yao
Tianjin Key Laboratory of Composite and Functional Materials, School of Materials Science and Engineering, Tianjin University, Tianjin 300072, People's Republic of China
e-mail: jpzheng@tju.edu.cn

C. Wang · J. Shi
Department of Prosthodontics, Stomatological Hospital, Tianjin Medical University, Tianjin 300070, People's Republic of China

preclinical evaluation of biocompatibility of PMMA/MMT nanocomposites, which can limit its application as denture base material.

As new kinds of denture base material, there are many biological factors that limit their application and development. For instance, residual monomer, resulted from incomplete monomer conversion in polymerization, has the potential to elicit inflammation, irritation, and allergic response of the oral mucosa [15]. Other substances like formaldehyde released from acrylic denture base could also cause adverse reactions [16]. Moharamzadeh [17] measured the amounts of monomers released from composite resins based on different monomer types by HPLC analysis. Many factors in the oral environment such as thermal variation, chemical and mechanical degradation affect the release of components from dental materials [18, 19]. Moharamzadeh [20] has described a full-thickness tissue engineered human oral mucosal model for biological assessment of dental biomaterials. There are several reports on in vitro cytotoxicity tests used as one of the criterions for biocompatibility [21, 22]. However, according to ISO 10993, ISO/TR 7405-1997 and so on [23–25], the standard concerns the preclinical testing of dental materials used in dentistry in many aspects, including in vivo and in vitro [26]. To the best of our knowledge, there are no reports on overall preclinical evaluation of biocompatibility of the PMMA/MMT nanocomposites as denture base material.

In this paper, PMMA/MMT nanocomposites were prepared by in situ suspension polymerization with MMT modified by dodecylamine. The structure of the nanocomposites was characterized by means of X-ray diffraction (XRD) and transmission electron microscopy (TEM). The preclinical testing were used to evaluate the biocompatibility of PMMA/MMT material, including cytotoxicity test, hemolysis test, acute systemic toxicity test, oral mucous membrane irritation test, guinea-pig maximization test and mouse bone-marrow micronucleus test.

2 Experimental

2.1 Materials

Methyl methacrylate (MMA) was purchased from Tianjin Chemical Reagent Institute (Tianjin, China) and purified by the standard treatment with 5% aqueous NaOH followed by distillation at a normal pressure and then stored at low temperature before use. Sodium MMT was supplied by Huate Chemical Co. (Zhejiang, China). All animal related procedures were in accordance with the principles of laboratory animal care. All other reagents were commercially available and used as received.

2.2 Instrumentation

To measure the change in the gallery distance of MMT before and after intercalation, XRD was conducted utilizing a Japan Rigaku D/MAX-2500 diffractometer with Cu-Kα radiation ($\lambda = 0.154$ nm) at a power of 40 kV $\times$ 100 mA. The microstructure of the nanocomposites was characterized by TEM, using HITACHI-800 TEM with 200 kV accelerating voltage. The samples for TEM were cut to 60 nm thick sections with a diamond knife.

2.3 Synthesis of PMMA/MMT nanocomposites

2.3.1 Modification of the MMT

MMT were modified by dodecylamine before suspension polymerization. A typical modified method was carried out as follows: the mixture of 2 g of dodecylamine and 50 ml of distilled water was dropwise added to 200 ml 2 wt% ultrasonically pretreated MMT suspension under stirring at 80°C. The reaction was carried out for 1 h to obtain modified MMT.

2.3.2 Preparation of PMMA/MMT nanocomposites

PMMA/MMT nanocomposites were prepared by in situ suspension polymerization. A typical synthetic process was described as follows: the mixture of modified MMT and MMA monomer (mass ratio: 1:100) were dispersed ultrasonically and then diluted with distilled water containing hydroxyethyl cellulose and sodium dodecyl sulfate, stirred until obtaining homogeneous suspension system. The mixture was poured into the suspension polymerization reactor and then heated to 75°C under a nitrogen atmosphere for 30 min. Then 1 wt% (based on MMA) benzoyl peroxide (BPO) initiator was dispersed into the system, the reaction was performed for 12 h. The result was repeatedly washed with water, followed by filtered and then dried in a vacuum oven overnight at 60°C to obtain PMMA/MMT nanocomposites.

2.4 Cytotoxicity tests

2.4.1 Cell line, culture medium, reagents and equipment

Vero cell [27] (CCL81, Microbiology laboratory, Tianjin Medical University, Tianjin, China) were cultured in minimum essential medium, supplemented with 10 wt% fetal bovine serum (FBS, Institute of Hematology, Chinese Academy of Medical Sciences, Tianjin, China), containing penicillin (100 U/ml), streptomycin (100 U/ml) and 1 wt%

glutamine. Eagle's Basal Medium, containing 2.2 g/l sodium bicarbonate, 3.0 g/l HEPES and 50 ml/l FBS. Cell nutrition media was made by 10 wt% FBS, 1 wt% glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Cell maintenance media were made by 2 wt% FBS, 1 wt% glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Versene solution was made by 0.2 g ethylene diamine tetraacetic acid (EDTA), 8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$ and 0.2 g KH_2PO_4 and distilled water to 1,000 ml. The tetrazolium salt, MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma Co. (America), was dissolved in phosphate-buffered saline solutions (PBS, pH = 7.4) at a concentration of 5 mg/ml and stored at 4°C immediately before use. Dimethyl sulfoxide (DMSO) was also purchased from Sigma Co. (America).

2.4.2 Sample preparation

According to material-forming processing of denture base materials, PMMA/MMT nanocomposites and common dental base acrylic resin powder supplied by Shanghai Dental Material Co. (Shanghai, China) were introduced into the resin dough. The dough were put into the mould between two polyester films and pressed under a pressure of 30 MPa in a hydraulic pressing apparatus. The mold was placed in water and heated to 70°C, and this temperature was maintained for 90 min. The temperature of the water was then increased to 100°C and kept at this temperature for 1 h. The test specimens were removed from the mold after cooling to room temperature. Samples (Fig. 1) of the common PMMA denture base material and the PMMA/MMT nanocomposite denture base material were prepared by cutting segments from the test specimens with a diamond cutting disk. Afterwards the corners were ground with grinding papers to make cylindrical samples with a diameter of (6.0 ± 0.2) mm and a height of (2.0 ± 0.2) mm [28]. Sample A stands for common denture base material and sample B stands for PMMA/MMT nanocomposite denture base material.

2.4.3 MTT

The samples were placed into the test tubes for getting the extracts, mixing with cell maintenance media and placed into a water-saturated atmosphere at 37°C for 24 h. The specimens were removed and the extracts were filtered using Millex GS sterile filters (Millipore, France). The positive reference was 1‰ phenol solution and the negative reference was cell maintenance media. The MTT test was carried out according to Edmondson et al. [29]. MTT was reduced by mitochondrial succinate dehydrogenase to yield a blue formazan product, which cannot cross the plasma membrane and it is accumulated in the cells. The cell membrane was then lysed by adding the Versene solution, releasing the dissolved formazan product, determined using a spectrophotometer.

Cells were incubated in 96-well cell culture clusters, with 100 µl extract for 24 h. All wells were aspirated and 100 µl extract or medium (control) was added, eight samples for each group. The samples were tested with enzyme-linked immunosorbent assay reader (ELISA reader, Bio-tek, America) at 492 nm wavelength at 2, 4 and 7 days, and the cells were observed under an inverted phase-contrast microscope (IMT-2, Olympus, Japan). The results from the experiments were statistically analyzed by one-way ANOVA using SPSS Microsoft. The results from each group within the same experiment were compared. Cytotoxicity was rated based on cell viability, represented by relative growth rate (RGR) of the cells (RGR = experimental group/negative control group × 100%), and then transfer to cytotoxicity grade (CG) ranging from 0 to 5.

2.5 Hemolysis test

Hemolytic activity was assessed by determining hemoglobin release under static conditions using the two-phase ISO/TR 7405-1984 hemolysis test [30, 31].

The samples were treated in the same way mentioned in Sect. 2.4.3. The positive reference was distilled water and

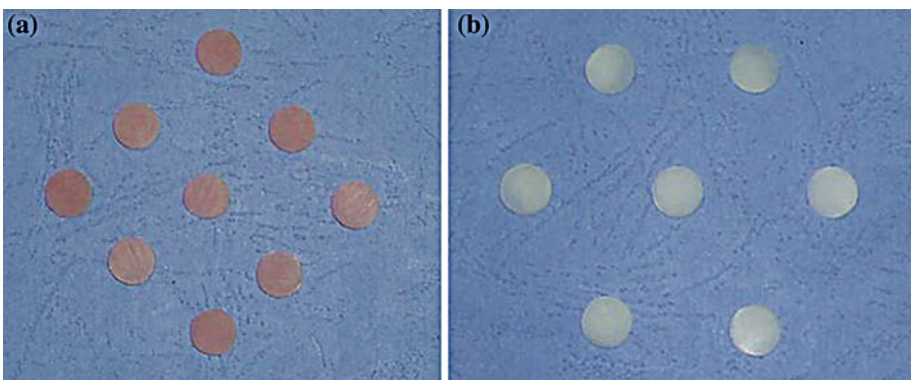

Fig. 1 Samples of the common PMMA denture base material and the PMMA/MMT nanocomposite denture base material: **a** common PMMA denture base material (Sample A), **b** PMMA/MMT nanocomposite denture base material (Sample B)

the negative reference was 0.9% physiological saline. Blood testing solution was prepared by using 20 ml fresh rabbit whole blood with 1 ml of 2% potassium oxalate, and took 8 ml of the treated blood then diluted with 10 ml of 0.9% physiological saline. In the first phase, each sample was incubated at 37°C for 30 min in pure saline, then diluted rabbit blood was added and incubation went on for another 60 min. In the second phase, the immersion liquid was centrifuged at 2,500 rpm for 5 min and the topical density of the supernatant was read by an ultraviolet spectrophotometer at 545 nm. The extent of hemolysis is manifested as hemolysis rate (%) using the following equation:

$$\text{Hemolysis rate} = \frac{\text{O.D}_{\text{sample}} - \text{O.D}_{\text{negative}}}{\text{O.D}_{\text{positive}} - \text{O.D}_{\text{negative}}} \times 100\% \qquad (1)$$

where O.D sample is the optical density values of adsorbent sample, O.D positive is the optical density values of adsorbent positive control and O.D negative is the optical density values of adsorbent negative control.

2.6 Acute systemic toxicity test

Ten male and ten female (nulliparous and non-pregnant) KunMing rats were approximately 9–10 weeks old on the day of dosing and then fasted approximately 18 h prior to dosing. The material samples were prepared in the same way mentioned in Sect. 2.4.2. The normal saline maceration extract of the sample-material was administered to rats in a single oral dose by gavage at the dose of 15,000 mg/kg body weight. Food was returned to the animals approximately 3–4 h after dosing. Individual weights were determined at the time of fasting (Day 1), shortly before the test substance was administered (Day 0), and on test days 1–7. The animals were observed for clinical signs of toxicity while handled before and after fasting, once during the first 30 min after dosing, at least two more times within 4 h after dosing, and daily thereafter. Observations for mortality and signs of illness, injury, or abnormal behavior were conducted twice daily [32, 33].

2.7 Oral mucous membrane irritation test

Healthy young adult Golden hamsters of either sex from a single outbred strain are used and they are acclimatized and cared for as specified according to ISO 10993-2 [34]. Remove the collar from each animal and evert the cheek pouches. The material samples were prepared in the same way mentioned in Sect. 2.4.2. Place both PMMA/MMT samples and normal samples with diameter 5 mm and thickness 1 mm, directly into the cheek pouch. No sample in placed in the other cheek pouch, which serves as blank control. Appropriate control animals also were tested in parallel. Examine the pouches macroscopically and describe the appearance of the cheek pouches for each animal and grade the pouch surface reactions of erythema for each animal at each time interval [35]. Record the results for the test report. At 2 weeks after the treatment, humanely sacrifice the hamsters and remove tissue from representative areas of the pouches. Place in an appropriate fixative prior to processing for histological examination.

2.8 Guinea-pig maximization test

Male albino guinea pigs were used to assess the dermal contact sensitization potential of PMMA/MMT denture material. The maximization test was conducted according to ISO 10993-10 [35]. The material samples were prepared in the same way mentioned in Sect. 2.4.2. On the first day of the induction period the scapular region of animals was clipped and three pairs of intradermal injections (0.1 ml/site) were made: Freund's Complete Adjuvant; sample preparation and a 1:1 mixture of Freund's Complete Adjuvant with sample preparation. Dermal irritation reactions were monitored and scored for a period of 3 days. On day 7 the area between the injection sites was re-clipped and rubbed with 10% sodium lauryl sulfate to provoke a mild inflammatory reaction. The following day this same area was treated with sample preparation on filter paper and held in place for 48 h using tape and an elastic bandage. The positive control animals were administered 10% dinitrochlorobenzol and the negative control animals were administered physiological saline in place of sample preparation. The first challenge was conducted 22 days after the initial injection. One flank of each of the animals was treated in the same way and held in place for 24 h. The treated sites were clipped and washed and assessed for challenge reactions 24 and 48 h after removal of the dressing. A similar re-challenge was done on the contralateral flank approximately 1 week after the first challenge [36–38].

2.9 Mouse bone-marrow micronucleus test

The mouse bone-marrow micronucleus assay was conducted following the recommendations of Willi Suter and MacGregor [39, 40]. Young adult KunMing mice were kept under standard animal room conditions (12 h light and 12 h dark circle, 21–25°C, relative humidity 20–49%, water ad libitum) and fed certified standard diet ad libitum up to 12 h before dosing. The material samples were prepared in the same way mentioned in Sect. 2.4.2.

In a dose range-finding experiment, doses of 15, 7.5, 3.75 g/kg body weight small particles of sample-material, dissolved in physiological saline, were given once by oral gavage. Positive control animals received 40 mg/kg

cyclophosphamide per oral gavage and were sacrificed 24 h after treatment; negative ones were treated in the same way but using physiological saline in place of cyclophosphamide.

Bone-marrow smears were prepared from femurs and stained with May–Gruenwald Giemsa. Two slides per animal were prepared and 1,000 polychromatic erythrocytes (PCE) per slide were analyzed for the presence of micronuclei. To determine the ratio of PCEs to normochromatic erythrocytes (NCE) at least 1,000 erythrocytes were analyzed per slide. The data were analyzed using the Chi-squared-Contingency-Test ($F = 1$, $P < 0.05$).

3 Results and discussion

3.1 Preparation of PMMA/MMT nanocomposites

Figure 2 shows the XRD patterns of original MMT, modified MMT and PMMA/MMT nanocomposite. The sharp peak corresponding to the (001) plane of the original MMT appears at 7.06°. According to the Bragg's equation: $\lambda = 2d \sin\theta$, the d_{001} is 1.25 nm. The sharp peak corresponding to the (001) plane of the modified MMT appears at 4.98°, the d_{001} is 1.77 nm, and no diffraction peak at $2\theta = 2$–10° corresponding to the (001) plane of MMT can be observed from the XRD pattern of PMMA/MMT nanocomposite. The results show that the layer distance of MMT was increased and reveal that the modifier has intercalated into the sheets of MMT. The absence of the d_{001} peaks of PMMA/MMT nanocomposite implies that the layered ordered structure of modified MMT was destroyed when the PMMA molecular chains grew in the layers of modified MMT and the exfoliated nanocomposites were formed during the polymerization.

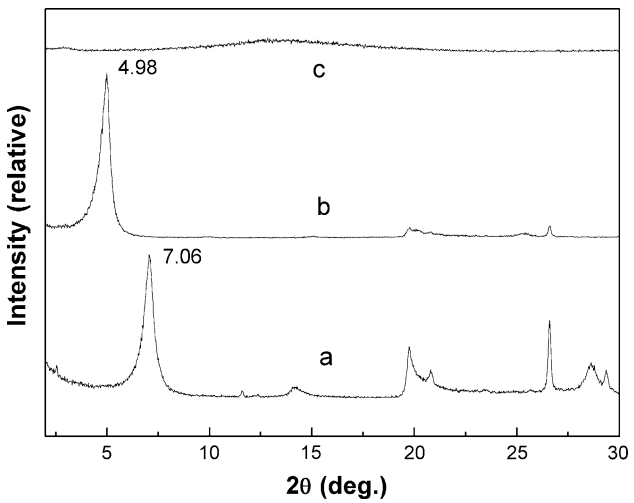

Fig. 2 XRD patterns of original MMT, modified MMT and PMMA/MMT nanocomposite: **a** original MMT, **b** modified MMT, **c** PMMA/MMT nanocomposite

Fig. 3 TEM image of PMMA/MMT nanocomposite

TEM has proven to be a powerful tool for studying the dispersion and microstructure of nanofillers embedded within a polymer matrix. Figure 3 shows the TEM image of PMMA/MMT nanocomposite, in which the bright field represents the PMMA matrix and the dark lines stand for the MMT layers. Figure 3 shows that the MMT layers are well dispersed and the well order of MMT layers has disappeared, the layered ordered structure of modified MMT was exfoliated into secondary particles. Therefore, these results indicate that an exfoliated PMMA/MMT nanocomposite has been prepared.

3.2 Cytotoxicity tests

MTT was reduced by mitochondrial succinate dehydrogenase to yield a blue formazan product, which cannot cross the plasma membrane and it is accumulated in the cells. The cell membrane was then lysed by adding the Versene solution, releasing the dissolved formazan product, determined using a spectrophotometer [31]. Figure 4 shows the phase-contrast microscope photographs (400× magnification) of the cells of all experimental groups at the seventh day. According to the photographs, the cell viability percentages of positive control group is obviously lower than the others groups, but the cell viability percentages of the negative one, sample A and sample B are almost the same. So there is significant difference between positive control group and the others, but no difference among the negative one, sample A and sample B.

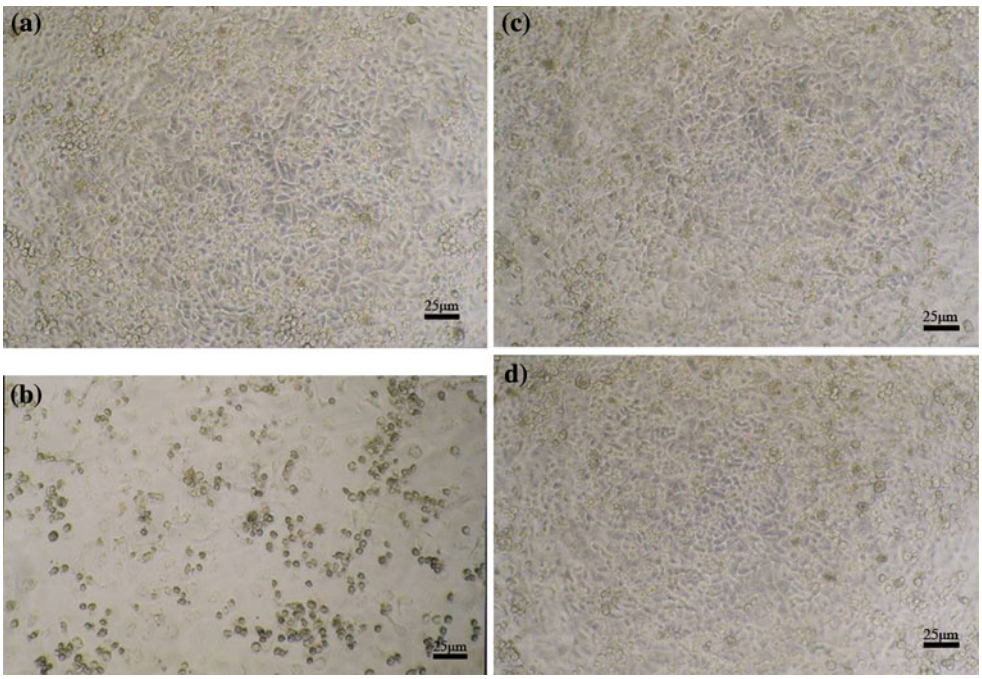

Fig. 4 Phase-contrast microscope photographs (400× magnification) of the cells of all experimental groups at the seventh day: **a** the negative reference group, **b** the positive reference group, **c** the group of sample A, **d** the group of sample B

Cytotoxicity was rated based on cell viability, represented by relative growth rate (RGR) of the cells (RGR = experimental group/negative control group × 100%), and then transfer to cytotoxicity grade (CG) ranging from 0 to 5 [41]. Absorbance of all experimental groups is presented in Table 1. Table 2 shows cell relative growth rate (RGR) and cytotoxicity grade (CG) of all experimental groups. Based on Table 1, there is also statistically significant difference between positive control group and the others, but no difference between the negative one and the others. There was no significant difference between the PMMA/MMT nanocomposites and the common PMMA denture resin material ($P > 0.05$) and the cytoxicity grades (CG) of the two kinds of material are one. So PMMA/MMT nanocomposites can be classified as non-cytotoxic material.

Table 1 Absorbance of all experimental groups of cytotoxicity tests

Sample number of cases		Absorbance		
		2d	4d	7d
Negative	8	0.560 (0.028)	0.648 (0.013)	0.749 (0.017)
Positive	8	0.340 (0.027)	0.236 (0.019)	0.124 (0.013)
A	8	0.555 (0.028)	0.645 (0.022)	0.735 (0.015)
B	8	0.546 (0.024)	0.644 (0.024)	0.734 (0.016)

Table 2 Cell relative growth rate (RGR) and cytotoxicity grade (CG) of all experimental groups

Sample	2d		4d		7d	
	RGR	CG	RGR	CG	RGR	CG
Negative	100	0	100	0	100	0
Positive	60.7	2	36.4	3	16.6	4
A	99.1	1	99.5	1	98.1	1
B	97.5	1	99.4	1	98.0	1

3.3 Hemolysis test

Table 3 presents the Hemolysis rate of PMMA/MMT nanocomposite and control groups. Based on Table 3, the Hemolysis rate of the sample-material was lower than 5%, and according to ISO/TR7405-1984(E), the result of hemolysis experiment was up to standard [31].

Table 3 The hemolysis rate of PMMA/MMT nanocomposite and control groups

	Sample B O.D	Negative O.D	Positive O.D	Hemolysis rate (%)
1	0.056	0.021	0.896	4.00
2	0.049	0.019	0.793	3.88
3	0.053	0.024	0.859	3.47

3.4 Acute systemic toxicity test

The median lethal dose (LD_{50}) was used to determine the acute toxicity as described in methods. The toxic response such as severe prostration, apathy, respiratory distress, and catatonia may be observed and several mice may die after 24 h [42]. But in our test, no obvious changes were observed, all animals gained weight and no adverse clinical signs and no death were observed in rats over the 7 days following oral administration at a dose of 15,000 mg/kg. LD_{50} was more than 15,000 mg/kg. Additionally, no gross lesions were found in any of the organs at necropsy. According to the standard of acute toxicity grade, the suspended liquid tested is not acutely toxic.

3.5 Oral mucous membrane irritation test

Macroscopic evaluation: The animals' exercise and diet was normal. There was no abnormal reaction on local mucous membrane such as hyperemia, haemorrhage, erosion, ulcer and so on. The pouch surface reactions for erythema were absent. There was no significant difference with the negative control. Figure 5 shows the tissue slicings used for histological examination using HE staining (100× magnification). Histological evaluation showed that the epithelium was normal, cells stratifications were clear, the permutations were well-distributed. Hypodermis was loose, the forms of glands were regular, and there was no inflammation cell infiltration in connective tissue. All these had no significant difference with tissue slicings of the negative control group.

3.6 Guinea-pig maximization test

Skin reactions in both the irritation and challenge portions of the study were graded on a 0–4 scale, with zero indicating no reaction and four representing severe erythema with slight eschar formation or severe edema. There was no patho-change on the skin of tested sample-material and the negative contrast, but the skin of positive contrast showed obvious oedema and middle or serious erythema in 24 h after stimulated, and later showed erosion and scab. The tested sample-material caused isolated grade 1 skin reactions and can be classified as weak allergens.

3.7 Mouse bone-marrow micronucleus test

Table 4 presents the results of micronucleus test of all experimental groups. According to Table 4, there was no significant difference between female and male mice's micronucleus ($P > 0.05$), and between every dose group of tested sample-material and the negative contrast ($P > 0.05$). However, there is difference between the positive and the negative control ($P < 0.01$). The sternum marrow cells formation (PCE/NCE) of this experiment was normal and obvious inhibition was found. The micronucleus studies correspond very well in test protocols used and the negative results obtained.

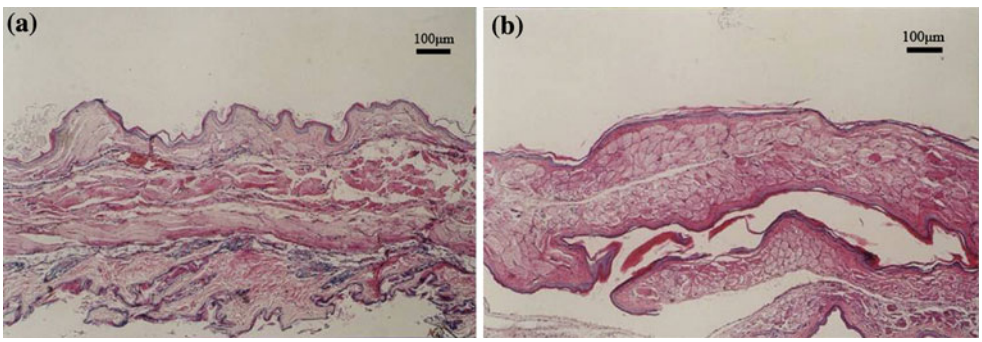

Fig. 5 The tissue slicings used for histological examination using HE staining (100× magnification): **a** the group of sample A, **b** the group of sample

Table 4 The results of micronucleus test of all experimental groups

Sample	PCE	Micronucleus number	Micronucleus rate (‰)	χ^2	*P*
Negative	10069	25	2.48	N/A	N/A
3.75 g/kg	10033	15	1.50	2.469	0.153
7.50 g/kg	10223	26	2.54	0.007	1.000
15.0 g/kg	10124	28	2.77	0.154	0.784
Positive	10081	442	43.84	380.64	0

4 Conclusion

PMMA/MMT nanocomposites were successfully prepared by in situ suspension polymerization, and the preclinical evaluation of biocompatibility as denture base material was also investigated. XRD and TEM results reveal that an exfoliated nanocomposite was achieved. Due to the results of cytotoxicity test, hemolysis test, acute systemic toxicity test, oral mucous membrane irritation test, guinea-pig maximization test, and mouse bone-marrow micronucleus test, PMMA/MMT nanocomposites have excellent biocompatibility and complies with the International Standard for denture base material and have potential application in dental materials.

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