Porous phosphate-gelatine composite as bone graft with drug delivery function

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The design and synthesis of porous phosphate-gelatine composite implant which mimicks the structure of natural bone and has drug delivery function is proposed.

Gelatine reproducing the proteinaceous part of bone was cross-linked in order to modulate its solubility in the physiologic fluids. The kinetic of gelatine release from ceramic matrix was also evaluated as model of the release of any therapeutic compound which can be loaded into gelatine.

1. Introduction

The scarcity of bone tissue is one of the major problem orthopaedic surgeons have to face in the prosthetic interventions and particularly in the revision prosthesis operations whose incidence is growing up in recent years. The necessity to replace a portion of the lost bone tissue has induced the development of specific biomaterials, so to avoid the necessity of drawing autologous bone, an operation involving a longer surgical procedure and the possibility of postoperative complications.

The properties required by a biomimetic composite material are biocompatibility, biodegradability, ability to initiate osteogenesis, composition and mechanical properties similar to those of natural bone. All these features are present in biological polymers like gelatine and collagen linked to a mineral phase based on Ca-phosphates compounds.

Porous HA is a suitable framework for the organization of cells of bone tissue, indeed several studies reported that Ca-phosphate compounds provide a scaffold for the ingrowth of bone with no evidence of adverse response [1–4]. However, the brittleness of porous material and its tendency to form debris sometimes limited the applications. Therefore, studies are now devoted to composite material (hydroxyapatite/proteinic polymers) that show an excellent balance between strength and toughness and contemporarily can act as reservoir for the release of substances like drugs, growth factors, etc.

A positive contribution to cell growth has been recently found in scaffolds consisting mainly of gelatine [5]; additionally some studies have been carried out on glutaraldehyde as cross-linking agent which is added to gelatine to control its solubility kinetic, and also for this agent a good tolerance by cells has been confirmed [6–8].

Together with the fundamental role as a bone tissue substitute, the importance to have a local therapy through drug delivery action has to be emphasized; in fact, local drug delivery could represent a more effective and less costly approach to therapy of bone disease or of inflammation process in the prosthesis revision operation [9–12].

The aim of this study was to design a composite material for bone grafting, formed of an osteoconductive inorganic phase (HA) and an organic phase (gelatine) similar to that of bone.

Following the attainment and characterization of the composite implant, the kinetic of gelatine release was studied as a simulation of a typical drug release.

2. Experimental

2.1. Porous HA ceramics

HA powder was prepared by a precipitation technique starting from $Ca(OH)_2$ and H_3PO_4. Controlling precipitation temperature and ripening time, powders with different crystallinity degree were obtained. The details of the preparation have been reported in a previous paper [13]. Porous bodies were prepared by soaking cellulose sponges (Spontex) slightly humidified with slurry obtained by the HA ceramic powder. The slurry was in turn prepared by mixing the powder with a solution of distilled water (in the ratio 1 : 1 solid/H_2O) and a dispersant agent (Dolapix CA Zschimmers and Schwartz in the amount of 1%). Finally an ultrasonic treatment was applied.

Once the sponges were soaked, they were left to decant and dry in air for 72 h and then sintered at 1250 °C

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for 1 h in flowing air to allow a good emission of organic combustion products.

Raw materials, powders and sintered bodies were characterized microstructurally by X-ray diffraction (XRD) (Rigaku, Cu Kα radiation) and SEM (Leica, Cambridge), equipped with EDS microprobe analyzer (Link).

Mercury porosimetry was used to evaluate pore size distribution ($< 50\,\mu m$) by two different apparatus (Carlo Erba Porosimeter 2000 and Macropores Unit 120) working on separate pore size range (microporosity 0.018–7.5 μm and macroporosity 10–1000 μm). Specific surface area was evaluated by BET method with a Carlo-Erba Sorpty 1750 instrument.

Density of porous bodies was measured by Archimedes' method and by geometrical weight/volume evaluation.

The compressive strength was measured on cylindrical specimens (about 9.0 mm × 9.5 mm, diameter × height, respectively) using a universal testing machine Instron mod. 1195. A paper sheet was inserted between each flat surface of the specimen and the loading anvil to avoid friction effects. The specimens were loaded up to fracture with a constant crosshead speed of 0.5 mm/min.

2.2. Porous ceramic-gelatine composite

Type A gelatine (Italgelatine S.p.A.) from pig skin was used. Aqueous gelatine solutions at different concentration (2.5%, 5.0% and 10.0% w/w) were used to coat the previously prepared porous HA ceramics. The rod shaped ceramics samples were soaked in the gelatine solution while a slow suction under vacuum were applied to gain a bulky plate. The synthetic porous HA-gelatine composites were obtained after water evaporation at room temperature. After air drying some composites were cross-linked with glutaraldehyde (GTA) solutions at different concentrations, from 0.01 to 2.5% (w/w), in phosphate buffer at pH 7.4 for 24 h at room temperature. The cross-linked samples were then repeatedly washed with 0.1 M glycine water solution and then with bi-distilled water and air dried at room temperature to eliminate GTA excess.

The extent of cross-linking of the gelatine coating inside the porous HA bodies was determined by a UV assay of uncross-linked ε-amino groups before and after cross-linking [14]. Following reaction with 0.5% TNBS (2,4,6 Trinitrobenzenesulfonic-acid) [14], gelatine was hydrolyzed with 6 M HCl, and extracted with ethyl ether. The absorbance of the diluted solution was measured at 346 nm in a Kontron Uvikon 931 spectrophotometer against a blank. The relationship between absorbance and moles of ε-amino groups per gram of gelatine is:

$$\frac{\text{moles of } \varepsilon\text{-amino groups}}{\text{g gelatin}} = \frac{2(\text{absorbance})(0.020\,\text{L})}{(1.46 \times 10^4\,\text{L/mol cm})(b)(x)}$$

where 1.46×10^4 L/mol cm is the molar absorptivity of TNP- lys, b is the cell path in cm, x is the sample weight in grams and 0.020 L represents a dilution factor.

Each sample of porous HA-gelatine composite was immersed in 10 mL of a phosphate buffer solution, pH 7.4 at 37 °C for period of time ranging from 1 h to 6 months. Gelatine concentration in the release buffer was determined by colorimetric assay using a bicinchoninic acid protein assay kit (Sigma Chemical Co., St. Louis, MO, USA). A 4% copper(II) sulfate pentahydrate solution was mixed with an excess of bicinchoninic acid at a final ratio of 1 : 50 v/v; 200 μL of the release solution was added to 2 mL of the assay solution in a test tube. Following further addition of phosphate buffer solution up to a final volume of 5 mL, the solutions were stored at 37 °C for 30 min and then cooled to room temperature and the absorbance of each solution at 562 nm was measured using a Kontron Uvikon 931 spectrophotometer. The gelatine concentration in the release solution was determined through comparison with a calibration curve. Each experiment was performed in triplicate.

Since GTA toxicity seems to be related to its release from the biomaterial, the release of GTA from cross-linked samples of porous HA-gelatine composite (washed with 0.1 M glycine solution) was determined by means of a HPLC method through comparison with a calibration curve.

Morphological investigation of fracture surfaces of porous HA ceramics after soaking treatment with gelatine, was performed using an optical microscope (Wild Mikroscope MPS050). In order to distinguish the gelatine from HA the pellet was soaked in a phosphate buffer solution (pH 7.4) containing 0.67% of genipin. The samples were cut in the middle and the inner surface examined.

2.3. *In vitro* tests

Human mesenchymal stem cells were cultured for 10 days in plastic wells containing porous HA pellets and HA-gelatin pellets. Adherent cells were counted by a haemocytometric chamber, using the Trypan blue dye exclusion test, after two step of enzymatic treatment (0.05% trypsin-0.02% EDTA, Gibco) for 5 min at 37 °C. The efficiency of the recovery of the cells after enzymatic treatment was evaluated by SEM and the recovery of the cells on the ceramic surface and into the macropores was recognized to be completed. Each experiment was done in triplicate and the results were expressed as the total number of adherent cells in HA and HA-gelatine samples compared with control (plastic well).

3. Results and discussion

3.1. HA inorganic component

By varying the synthesis temperature and ripening time HA powders with different crystallinity degrees (in the range 20–80%) can be obtained [15].

Previous experiments demonstrated that powder with crystallinity degree of about 80% yield macroporosity with 200–300 μm, which is essential for bone ingrowth [2] and contemporarily gives sufficient mechanical resistance to the struts and walls of pores: actually the powder specific surface area and mean particle size distribution play a crucial role in the impregnation procedure.

In Fig. 1 the morphology of the porous ceramic

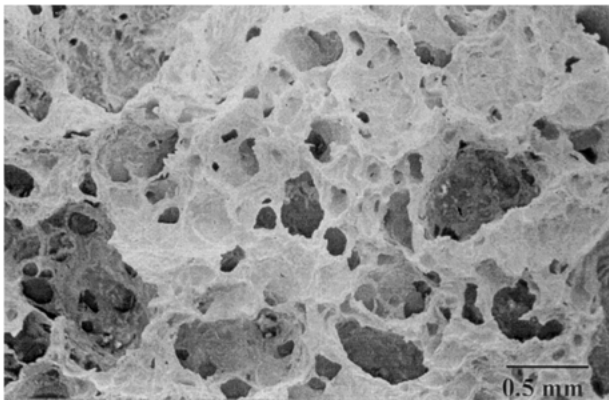

Figure 1 SEM micrograph of porous sintered HA body.

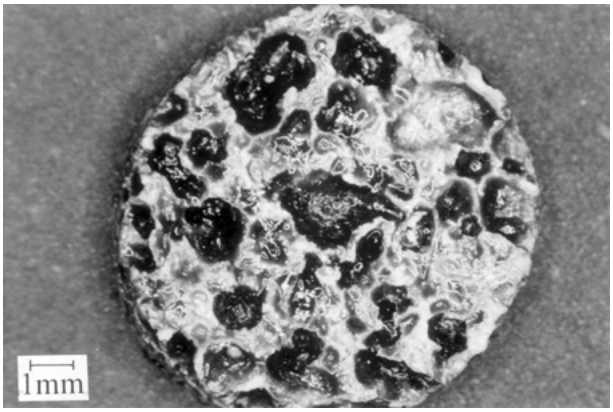

Figure 2 Optical micrograph showing the macro and micropores of the inner part of the composite where gelatin (blue colored by genipin) fills the inner part of the pores.

structure is reported showing macroporosity up to 400 μm and of a diffuse microporosity ranging between 10 and 0.1 μm.

Porosity values determined by geometrical method are included in the range 68–85%; the porosity determined by mean of porosimeter resulted 8–18% and 50–60% for microporosity and macroporosity, respectively. The sum of micro and macroporosity results lower than the total value determined by geometrical method due to the presence of small amount of closed porosity.

As concerns compressive strength all specimens broke in more or less ''porous'' fashion with a load peak followed by a progressive ''softening'' of the specimen. This progressive softening was more evident in the material characterized by the highest microporosity; the σ_{comp} varied between 5 and 8 MPa. Previous studies [13] found that in macroporous HA samples the compressive strength is inversely proportion to the microporosity of pore walls and struts and that the microporosity explains better than the total porosity the strength-porosity dependance: these findings well agree with the different values of σ determined for our porous materials.

The same measure was performed also on samples covered by gelatin and the value of σ_{comp} increased at 10–15 MPa; moreover the load-displacement curves show a more pronounced softening part, after the peak load, than those measured for inorganic porous materials without organic coating.

The pore interconnection was calculated according to the connectivity indicator proposed in Hing *et al*. [4]: the value was found to be ~ 4.

3.2. Proteinic coating

The amount of gelatine soaked in the porous ceramic sample increases as a function of the total porosity % of the inorganic sample as well as of the concentration of the gelatine solution in which the samples were soaked (gelatine solutions concentrated 2.5%, 5.0% and 10.0%). For example, the amount of gelatine determined in the dry porous ceramic-gelatine composites with $\sim 73 \pm 3\%$ of total porosity was 7–8%, 9–10% and 20–21% from gelatine solutions concentrated 2.5%, 5.0% and 10.0%, respectively.

The extent of gelatine penetration into the porous samples can also be determined by the evaluation of the blue colored area visible in Fig. 2 where the surface of an internal section of the porous sample after gelatine impregnation is showed. The blue color (which represents gelatine colored by genipin) is well visible inside the macro and micropores of the inner part of the sample proving that the gelatine penetrates the core of the sample which is in communication with the external surface.

In Fig. 3 the gelatine release (%) is plotted for uncross-linked ceramic-gelatine samples (with $73 \pm 3\%$ of total porosity) as a function of time, when solutions with different gelatine concentration (2.5%, 5% and 10%) is used for soaking.

The above reported results reveal that the highest amount and the best localization of the soaked gelatine inside the pores of the ceramic bodies is obtained when 10% gelatine solution is used; in this case the release appears continuous and more gradual for a longer period of time.

The results, plotted in Fig. 4 as a function of time, show a different trend of the gelatine release (%) for ceramics samples with $68 \pm 1\%$, $73 \pm 1\%$ and $85 \pm 1\%$ of total porosity when 10% soaking gelatine solution is used. The release trend was studied and found not satisfying the standard $t^{1/2}$ dependence. In this case the release does not follow the standard behavior found for tablets made by pressed powders, but it closely resembles an exponential function. We can hypothesize that the

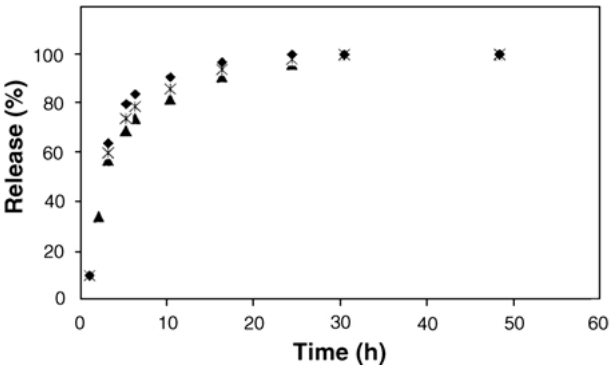

Figure 3 Gelatin release (%) is plotted for uncross-linked ceramic-gelatin samples (with $73 \pm 3\%$ of total porosity) as a function of time when solutions with different gelatin concentration (◆ 2.5%, ∗ 5% and ▲ 10%) is used for soaking.

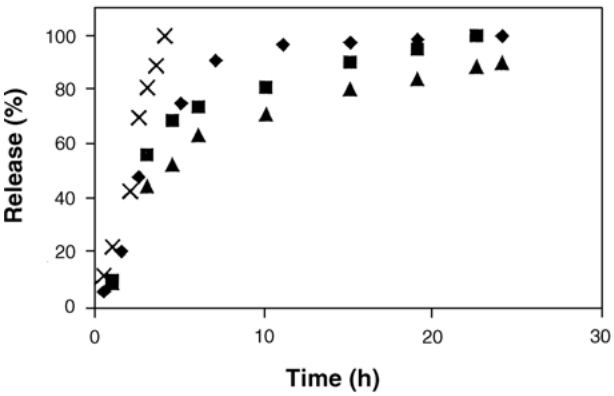

Figure 4 Gelatin release (%) for ceramics samples with 68 ± 1% (▲), 73 ± 1% (■) and 85 ± 1% (◆) of total porosity when 10% soaking gelatin solution is used. Gelatin film is used as control (X).

release is controlled by the diffusion coefficient of drug, pores dimensions and porosity interconnection extent of the ceramic matrix. All samples complete the 100% gelatine release within 30 h, but the higher is the % porosity of the ceramic samples the quicker is the bulky gelatine coat dissolution. Air dried gelatine bulk samples stored in physiological solution undergo quick solubility. In fact the gelatine samples dissolve in solution after 3 h (Fig. 4). The data put into evidence an interaction between gelatine layer and ceramic framework which prevents gelatine swelling and dissolution. Probably dimension, morphology and distribution of pores within the ceramic framework affect the protein swelling modifying the gelatine surface area exposed to physiological solution. In order to protract the dissolution time of gelatine we have taken into account the data previously reported [6] about the properties of gelatine films at different degrees of glutaraldehyde cross-linking. After air drying, gelatine samples were cross-linked with GTA solutions at different concentrations. The cross-linked samples, repeatedly washed with 0.1 M glycine water solution and then with bi-distilled water before air drying at room temperature, were stored in physiological solution.

3.3. Release kinetics

The gelatine concentration in the release buffer was determined in order to appreciate the dissolution kinetic of the gelatine films as a function of extent of cross-linking. Results reported in Fig. 5 show that gelatine films treated with 1 wt % GTA do not dissolve at all after 1 month in physiological solution. The values of gelatine release (%) of porous ceramic-gelatine composites treated with GTA solutions at different concentrations are reported in Fig. 6 as a function of time. Appreciable amounts of gelatine are released in buffer solution from samples cross-linked with GTA at lower concentration: up to about 30% after 30 days when samples are treated with 0.05% GTA solution. The degree % of gelatine release reduces as GTA concentration increases, down to 5% after 1 month and 100% after 10 months for samples cross-linked with 1 wt % GTA solutions. No gelatine release has been observed after 10 months of storage in

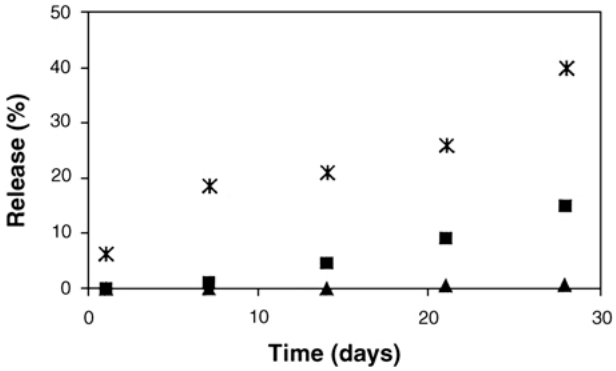

Figure 5 Gelatin release (%) is plotted for gelatin films cross-linked with different GTA concentrations (▲ 1 wt %; ■ 0.25 wt %; ∗ 0.05 wt %) as a function of time (days).

buffer solution for samples treated with GTA solution at concentrations > 1 wt %.

The GTA release was determined after different times of storage in buffer solution of samples cross-linked with GTA at different concentrations (Fig. 5). No GTA release was observed from samples cross-linked with solution at concentrations higher than 2 wt %. Samples cross-linked with GTA solutions at concentrations $\approx 2\%$ release after 2 days of storage in buffer solution an amount of GTA corresponding to about 1 wt %. The extent of cross-linking of gelatine bulky plate which cover the porous ceramics changes as a function of GTA concentration in the cross-linking solutions. The extent of cross-linking reach the 100% when 1.5–2.5% GTA solutions are used.

These results suggest that the phosphate-gelatine composites could have drug delivery function when any therapeutic compound is loaded into gelatine. Further experiments, now in progress, reveal that drugs with rod like molecular dimensions $\approx 12 \times 6$ are completely retained by gelatine, thus their release rate is controled by gelatine solubilization rate. Obviously in the case of drugs with smaller molecular size gelatine could behave as a sieve allowing a faster drug release.

The degree of cross-linking can be used to modulate the release kinetics in a wide range of time in accordance with the therapeutic application.

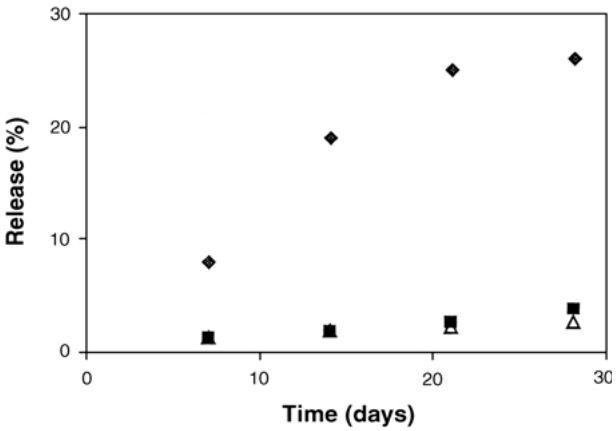

Figure 6 Gelatin release (%) is plotted for porous ceramic-gelatin composites cross-linked with different GTA concentrations (△ 1 wt; %; ■ 0.25 wt %; ◆ 0.05 wt %) as a function of time (days).

TABLE I Results of cell proliferation tests

	n cells	% versus control
Control	$5.36 \times 10^6 \pm 0.28 \times 10^6$	—
Porous HA	$5.56 \times 10^6 \pm 0.34 \times 10^6$	103.7
Porous HA + gelatine	$5.33 \times 10^6 \pm 0.11 \times 10^6$	99.5

3.4. Biocompatibility

Human mesenchymal stem cells proliferated on macropores of HA and HA-gelatine ceramics increasing in number by about 163% after 10 days of culture. After this period the number of cells was not statistically different on plastic, on HA and on HA-gelatine, indicating that ceramic and composite did not modify cell proliferation (see Table I).

4. Conclusions

Porous HA ceramics covered by gelatine was prepared as scaffold to replace natural bone: micro, macroporosity and porosity interconnection factor was controlled to resemble as much as possible the structure and morphology of human spongy bone tissue.

The amount of gelatine absorbed on the porous HA depends on the amount of porosity of the ceramic support and on the gelatine concentration in the water solution: 10% of gelatine was found to be the optimal concentration to assure a complete absorption and penetration into the bulk of the ceramic which in turn affects the gelatine swelling and assures its uniform release and protracted dissolution time. In order to further slow down the kinetic of gelatine release in buffer solution, GTA was used as cross-linking agent and its maximum concentration to avoid the release of GTA itself in SBF was determined to be 2%. Using GTA in the amount of 0.05% the kinetic release of gelatine slows-down from 100% of gelatine in 30 h to 30% of gelatine in 30 days. The gelatine release reduces as GTA concentration increases, down to 5% after 1 month and 100% after 10 months for samples cross-linked with 1 wt % GTA solution.

The biocompatibility tests demonstrate that the composite material included GTA do not modify cell proliferation. We can conclude that the porosity distribution and interconnection factor together with the amount of adsorbed gelatine can be designed to control the scaffold bioactivity which takes place through ion interchange between composite surface and phosphate buffer solution. On the other hand the concentration of the cross-linking agent allows the modulation of the delivery function in a wide range of time (gelatine being the vehicle of any drug which can be loaded inside), making the device extremely promising for various therapeutic applications.

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References

1. K. DE GROOT, *Ann. NY Acad. Sci.* **253** (1988) 227.
2. O. GAUTHIER, J. M. BOULER, E. AGUADO, P. PILET and G. DACULSI, *Biomaterials* **19** (1998) 133.
3. G. CAROTENUTO, G. SPAGNIUOLO, L. AMBROSIO and L. NICOLAIS, *J. Mater. Sci.: Mater. Med.* **10** (1999) 671.
4. K. A. HING, S. M. BEST and W. BONFIELD, *ibid.* **10** (1999) 135.
5. Y. SEON CHOI, S. RAN HONG, Y. MOO LEE, K. WON SONG, M. HYANG PARK and Y. SOO NAM, *Biomaterials* **20** (2000) 409.
6. A. BIGI, G. COJAZZI, S. PANZAVOLTA, K. RUBINI and N. ROVERI, *ibid.* **22** (2001) 763.
7. A. BIGI, B. BRACCI, G. COJAZZI, S. PANZAVOLTA and N. ROVERI, *ibid.* **19** (1998) 2335.
8. A. BIGI, M. BORGHI, G. COJAZZI, A. M. FICHERA, S. PANZAVOLTA and N. ROVERI, *J. Thermal Anal.* **61** (2000) 451.
9. J. A. BAKAN, in ''Poly Sci Tecnol 8'', edited by R. L. Kromethal, Z. Oser and E. Martin (Plenum, London, 1975) p. 213.
10. L. DI SILVIO, N. GURAV, M. V. KAYSER, M. BRADEN and S. DOWNES, *Biomaterials* **15** (1994) 931.
11. L. DI SILVIO, R. G. COURTENEY-HARRIS and S. DOWNES, *J. Mater. Sci.: Mater. Med.* **5** (1994) 819.
12. L. DI SILVIO and W. BONFIELD, *ibid.* **10** (1999) 653.
13. A. TAMPIERI, G. CELOTTI, S. SPRIO, A. DELCOGLIANO and S. FRANZESE, *Biomaterials* **22** (2001) 1365.
14. C. M. OFNER III and W. A. BUBNIS, *Pharm. Res.* **13** (1996) 1821.
15. A. TAMPIERI, G. CELOTTI, S. SPRIO and C. MINGAZZINI, *Mater. Chem. Phys.* **64** (2000) 54.

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