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(54) Titre : IMPLANT DE TISSU OSSEUX COMPRENANT DES IONS DE LITHIUM
(54) Title: A BONE TISSUE IMPLANT COMPRISING LITHIUM IONS

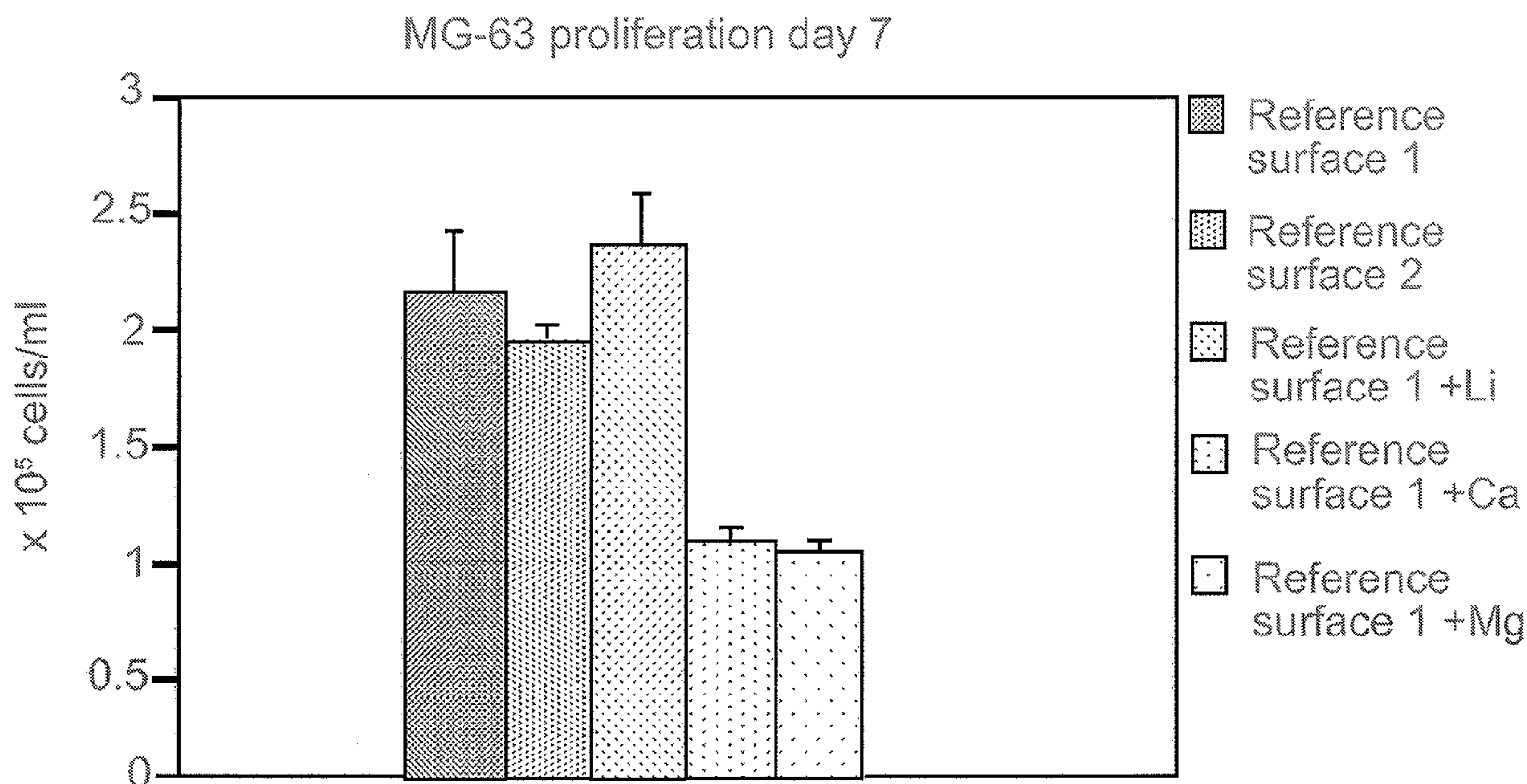


Fig. 11

(57) **Abrégé/Abstract:**

The present invention is based on that local administration of lithium ions in bone tissue has been found to improve the bone formation and bone mass upon implantation of a bone tissue implant in said bone tissue. In particular, the invention relates to a bone tissue implant having an implant surface covered by an oxide layer comprising lithium ions and a method for the manufacture thereof. A blasting powder comprising lithium ions, a method for locally increasing bone formation, and the use of lithium ions or a salt thereof for manufacturing a pharmaceutical composition for locally increasing bone formation are also provided by the present invention.

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(54) Title: A BONE TISSUE IMPLANT COMPRISING LITHIUM IONS

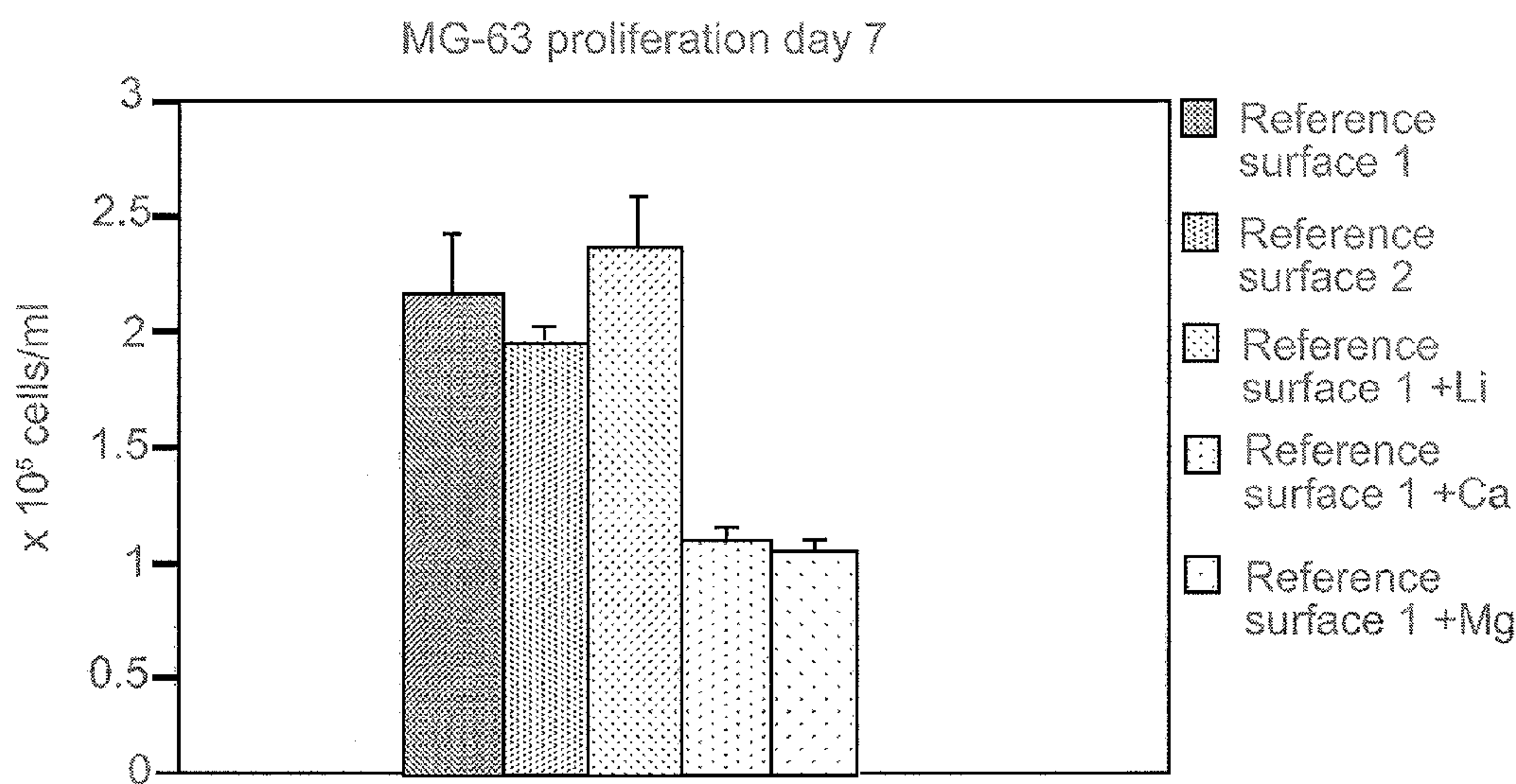


Fig. 11

(57) Abstract: The present invention is based on that local administration of lithium ions in bone tissue has been found to improve the bone formation and bone mass upon implantation of a bone tissue implant in said bone tissue. In particular, the invention relates to a bone tissue implant having an implant surface covered by an oxide layer comprising lithium ions and a method for the manufacture thereof. A blasting powder comprising lithium ions, a method for locally increasing bone formation, and the use of lithium ions or a salt thereof for manufacturing a pharmaceutical composition for locally increasing bone formation are also provided by the present invention.

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A BONE TISSUE IMPLANT COMPRISING LITHIUM IONS

Technical field

The present invention relates to an implant for implantation into bone tissue and a method for manufacturing thereof.

5 The invention also relates to a blasting powder and a method for locally increasing bone formation.

Technical background

10 A one-stage procedure is nowadays often used for implanting orthopaedic or dental implants, generally metallic implants, into bone tissue.

 In the one-stage procedure, a first implant part, such as a dental fixture, is surgically placed into the bone tissue, and a healing cap or a secondary implant part, such as an abutment, is then attached to the first implant part directly after the surgical operation. The soft tissue is thereafter
15 allowed to heal around the healing cap or the secondary implant part. When a healing cap is used, the cap is removed after a few weeks or months without any surgical procedure, and secondary implant parts, such as an abutment and a provisional crown, are attached to the first implant part. The one-stage procedure is for instance described in L Cooper et al: "A multicenter 12-month
20 evaluation of single-tooth implants restored 3 weeks after 1-stage surgery", The International Journal of Oral & Maxillofacial Implants, Vol 16, No 2 (2001).

 The two-stage procedure, which is another known implantation procedure, involves in a first stage surgically placing a first implant part, such
25 as a dental fixture, into the bone tissue, where it is then allowed to rest unloaded and immobile for a healing period of three months or more in order to allow the bone tissue to grow onto the implant surface to permit the implant to be well attached to the bone tissue, the cut in the soft tissue covering the implant site being allowed to heal over the implant, and in a second stage
30 opening the soft tissue covering the implant and attaching secondary implant parts, such as a dental abutment and/or a restoration tooth, to the first implant part, such as said fixture, forming the final implant structure. This procedure is for instance described by Brånemark et al: "Osseointegrated Implants in the

Treatment of the Edentulous Jaw, Experience from a 10-year period", Almquist & Wiksell International, Stockholm, Sweden.

However, the fact that the implant not should be loaded during the healing period means that the secondary implant parts may not be attached to the first implant part and/or used during the healing period of three months or more. In view of the discomfort associated with this, it is desirable to minimize the time period necessary for the above-mentioned first stage or even perform the entire implantation procedure in a single operation, i.e. to use the one-stage procedure.

For some patients, it might be considered better to wait at least three months before functionally loading the implant, both for one- and two-stage procedures. However, an alternative using the one-stage procedure is to put the implant in function directly after implantation (immediate loading) or a few weeks after implantation (early loading). These procedures are, for instance, described by D M Esposito, pp 836-837, in Titanium in Medicine, Material Science, Surface Science, Engineering, Biological Responses and Medical Application, Springer-Verlag (2001).

It is essential that the implant establishes a sufficient stability and bond between implant and bone tissue to enable the above disclosed immediate or early loading of the implant. It shall also be noted that an immediate or early loading of the implant may be beneficial to bone formation.

Some of the metals or alloys, such as titanium, zirconium, hafnium, tantalum, niobium, or alloys thereof, that are used for bone implants are capable of forming a relatively strong bond with the bone tissue, a bond which may be as strong as the bone tissue per se, sometimes even stronger. The most notable example of this kind of metallic implant material is titanium and alloys of titanium whose properties in this respect have been known since about 1950. This bond between the metal and the bone tissue has been termed "osseointegration" (Albrektsson T, Brånemark P I, Hansson H A, Lindström J, "Osseointegrated titanium implants. Requirements for ensuring a long-lasting, direct bone anchorage in man", Acta Orthop Scand, 52:155-170 (1981)).

It may be noted that in contact with oxygen, titanium, zirconium, hafnium, tantalum, niobium and their alloys are instantaneously covered with a thin oxide layer. This native oxide layer on titanium implants mainly consists of titanium(IV) dioxide (TiO_2) with minor amounts of Ti_2O_3 , TiO and Ti_3O_4 .

Other methods for obtaining a better attachment of the implant to the bone tissue involve alteration of the chemical properties of the implant surface.

Several methods involve the application of a layer of ceramic material, such as hydroxyapatite, to the implant surface, inter alia in order to improve the bonding of the implant to bone since hydroxyapatite is chemically related to bone. A disadvantage with coatings comprising hydroxyapatite is, however, that they may be brittle and may flake or break off from the implant surface, which may in turn lead to an ultimate failure of the implant.

Other methods for altering the chemical properties of the implant involve application of fluorine and/or fluoride on the implant surface (WO 94/13334, WO 95/17217, WO 04/008983, and WO 04/008984).

WO 2006/004297 discloses an osseointegrative metal implant, such as titanium or an alloy thereof, comprising a layer of metal oxide and a layer of a bio-active material composed of any one or more of Li, Na, K, Rb, Cs, Fr, Mg, Ca, Sr, Ba, Ra, Sc, Y, Lu, Ti, Zr, Hf, Nb, Ta, Cr, Mo, W, Mn, Re, Fe, Ru, Os, Co, Rh, Ir, Ni, Pd, Pt, Cu, Ag, Au, Zn, Ga, In, Sn and Bi formed thereon. A working example is, however, only described for a titanium implant comprising calcium as the bioactive layer.

Mention can also be made of WO 2002/096475 referring to a titanium implant comprising calcium, phosphor or sulphur in the titanium oxide layer, and WO 2005/084577 referring to a titanium implant comprising magnesium in the titanium oxide layer.

Although implants which provide a comparatively strong bond between the implant surface and the bone exist, there is a need in the art to enhance

Thus, there is a need in the art to provide an implant having a desired rate of attachment and which has the ability to form a mechanically strong bond between the bone and the implant upon implantation thereof in bone tissue.

It is an object of the invention to meet the above mentioned needs.

The inventors have found that lithium ions locally administered in bone tissue has a local effect on the bone formation and bone mass in the bone tissue.

Furthermore, it has been found that an implant comprising a surface oxide layer comprising and/or releasing lithium ions provides an increased proliferation of osteoblasts and an increased production of osteoprotegerin, in comparison to a metallic implant comprising a surface oxide layer containing, for instance, calcium or magnesium ions. An improved bone mass is thereby provided which implies a mechanically stronger bond between the implant and the bone tissue.

According to a first aspect of the invention, the above objects are achieved with an implant for implantation into bone tissue which has a surface covered by an oxide layer, wherein said oxide layer comprises lithium ions.

a) providing an implant having an implant surface;

- The method of the invention is inexpensive and easy to carry out,
5 thereby enabling mass production. Furthermore, it is easy to sterilize and to store.

According to a fourth aspect of the invention, a method for locally
10 increasing bone formation is provided. Said method comprises administering
a composition comprising lithium ions or a salt thereof and a pharmaceutically
acceptable carrier to a person in need thereof.

A fifth aspect of the invention relates to the use of lithium ions or a salt thereof for manufacturing a pharmaceutical composition for locally increasing bone formation.

A sixth aspect of the invention relates to a kit for implantation of an implant into bone tissue comprising an implant and a composition comprising lithium ions or a salt thereof and a pharmaceutically acceptable carrier.

Other features and advantages of the present invention will become
20 apparent from the following description of the invention.

Fig. 1 is a TOF-SIMS image illustrating the presence and the distribution of lithium for a sterilized titanium sample after reduction in LiNO_3 . (Lithium is shown as white dots.)

Fig. 2 illustrates the presence of lithium, (peak no 7) on a sterilized titanium sample (fig. 2a) compared to the reference sample of an oxalic acid treated sterilized titanium sample (fig. 2b) (from TOF-SIMS measurements).

Fig. 3 is a TOF-SIMS image illustrating the presence and the
30 distribution of lithium for a sterilized titanium sample after anodization in LiOH.

Fig. 4 illustrates the degree of proliferation of MG-63 cells after 3, 7 and 14 days culture on cell treated polystyrene with and without lithium in different concentrations.

Fig. 5 illustrates the production of alkaline phosphatase in the cell culture medium after 3, 7 and 14 days culture on cell treated polystyrene with and without lithium in different concentrations.

Fig. 6 shows the degree of proliferation of MG-63 cells on reference surfaces 1 and 2 after 7 days of culture with and without lithium in different concentrations.

5 Fig. 7 shows the production of alkaline phosphatase on reference surface 1 comprising lithium at a concentration of 1 mM compared to the unstimulated surface comprising no lithium.

Fig. 8 is a scanning electron microscopy image illustrating the morphology of MG-63 cells cultured on reference surface 1 after 36 h.

10 Fig. 9 is a scanning electron microscopy image illustrating the morphology of MG-63 cells cultured on reference surface 2 after 36 h.

Fig. 10 is a scanning electron microscopy image illustrating the morphology of MG-63 cells cultured on reference surface 1 comprising lithium after 36 h.

15 Fig. 11 illustrates the differences in MG-63 cell proliferation between reference surface 1, reference surface 2, and reference surface 1 comprising lithium, calcium and magnesium, respectively.

Fig. 12 shows the amount of osteoprotegerin measured after 7 and 14 days of cell culture on reference surface 1 comprising lithium, calcium, and magnesium, respectively.

20 Fig. 13 illustrates the removal torque test (RTQ) values after 6 weeks of implantation in rabbit tibia of an implant comprising lithium according to the invention compared to an implant with a commercially available Osseospeed™ surface.

25 Detailed description of the invention

As used herein the term “implant” includes within its scope any device intended to be implanted into the body of a vertebrate animal, in particular a mammal, such as a human. Implants may be used to replace anatomy and/or restore any function of the body.

30 Generally, an implant is composed of one or several implant parts. For instance, a dental implant usually comprises a dental fixture coupled to secondary implant parts, such as an abutment and/or a restoration tooth. However, any device, such as a dental fixture, intended for implantation may alone be referred to as an implant even if other parts are to be connected thereto.

As used herein the term “implant (intended) for implantation into bone tissue” refers to implants intended for at least partial implantation into bone

An example of an implant surface intended for implantation into bone tissue is the surface of a dental fixture that is intended for implantation into the jawbone of a patient and to be in contact with bone tissue.

The present invention relates to an implant for implantation into bone tissue having a surface; said surface being covered by an oxide layer, wherein said oxide layer comprises lithium ions.

An implant according to the invention provides an improved implant stability and bone tissue response as measured by removal torque (RTQ) tests (fig 13).

Lithium is a small, positively charged, non-toxic, light weight ion which has been found to be easily dispersed in the oxide layer covering the surface of the implant.

Although a therapeutic effect of lithium salts is known for manic-
35 depressive disorders, studies have shown that lithium therapy also affects the
bone mineral metabolism (for instance, Baran et al, "Lithium Inhibition of Bone
Mineralization and Osteoid Formation", J Clin Invest, pp 1691-1696 (1978);

Recently, oral administration of lithium chloride has been suggested for treatment of disorders of low bone mass, such as osteoporosis, since it has been concluded that lithium enhances bone formation and improves bone mass via activation of the canonical Wnt pathway resulting in, for instance, stimulation of pre-osteoblast replication, induction of osteoblastogenesis and inhibition of osteoblast and osteocyte apoptosis (Clément-Lacroix et al, “Lrp5-independent Activation of Wnt Signalling by Lithium Chloride Increases Bone Formation and Bone Mass in Mice”, PNAS, vol 102, No 48, pp 17406-17411 (2005) and Krishnan et al, “Regulation of Bone Mass by Wnt Signaling”, J Clin Invest, vol 116, No 5, pp 1202-1209 (2006).

The Wnt signalling pathway is an attractive target for the treatment of osteoporosis, and in general for bone anabolic drug discovery (Rawadi G et al, "Wnt signalling pathway: a new target for the treatment of osteoporosis", Expert. Opin. Ther. Targets, Vol 9, pp 1063-1077 (2005))). The fact that lithium activates the Wnt pathway makes the present invention highly suitable for implantation into bone tissue.

Furthermore, the fact that lithium previously has been used for the treatment of manic depressive disorders implies that the toxicological picture and the side effects upon systemic administration are well known.

25 In addition, lithium has a relatively simple chemistry and is generally indestructible and unaffected by e.g. sterilization.

The incorporation of lithium ions into the oxide layer may disrupt the oxide structure, thereby making the oxide more reactive. When the oxide layer is incorporated with positively charged lithium ions, an increased positive surface charge density is provided on the oxide surface (implant surface). Hence, electron rich proteins in the bone tissue may be electrically attracted to the surface. Incorporated ions can also affect the conductivity of the oxide which may have a positive effect on osseointegration and hemocompatibility.

35 At least part of the oxide layer should comprise lithium ions, and the desired osseointegrative effect of lithium in an implant of the invention may be achieved by the presence of the ions in the oxide layer. However, it may also

Preferably, lithium ions are homogenously distributed in the oxide layer. Such homogenous distribution is illustrated in figure 1.

In embodiments however, the implant can be a non-metallic implant comprising e.g. a ceramic, a plastic or a composite material. In such embodiments, the implant surface is a metallic implant layer applied on the non-metallic implant, whereby a partly metallic implant surface is provided. The metallic implant layer preferably comprises titanium or an alloy of titanium.

The oxide layer covering the surface of the implant has a thickness within the range of from 2 to 100 nm.

In preferred embodiments, the oxide layer is an oxide layer which is formed spontaneously, e.g. in contact with air. The thickness of such a spontaneously formed oxide layer is within the range of from 2 to 18 nm, for example within the range of from 2 to 6 nm.

Metal implants surfaces covered by oxide layers are known in the art. However, several prior art documents stress the importance of providing a thick oxide layer, preferably above 600 nm onto the implant surface (Sul et al, "Resonance frequency and removal torque analysis of implants with turned and anodized surface oxides", Clin. Oral. Impl. Res., Vol 13, pp 252-259 (2002); Sul et al, "Qualitative and quantitative observations of bone tissue reactions to anodised implants", Biomaterials, Vol 23, No 8, pp 1809-1817

(2002)). Such implants require an additional step of oxidation since oxide layers of the above mentioned thickness are not obtainable spontaneously.

The present inventors have found that an oxide layer having a thickness of less than 100 nm, preferably an oxide layer having the thickness of a native oxide layer, i.e. a spontaneously formed oxide layer, of less than 18 nm is more suitable for implantation into bone tissue since thick oxide layers may be very brittle. Furthermore, thick oxide layers may lead to cracking and peeling during longer periods of implantation of an implant in bone tissue.

10 This finding is in contrast to Xiropaidis et al who states that titanium implants with native oxide layers are considered less osteoconductive. (Xiropaidis et al, "Bone-implant contact at calcium phosphate-coated and porous titanium oxide (TiUnite™)-modified oral implants", Clin. Oral. Impl. Res, No 16, pp 532-539 (2005)).

15 An oxide layer according to the invention does not interfere with, or modify the topography of the implant surface. Furthermore, an implant comprising an oxide layer having a thickness of less than 100 nm, e.g. less than 18 nm, e.g. between 2 and 6 nm is biocompatible making it suitable for incorporation into the human body.

20 Hence, an oxide layer comprising lithium ions according to the invention is suitable for any geometry or any substrate.

The implant surface of the implant according to the invention is preferably a metallic implant surface comprising a metal oxide layer on its surface.

25 In particular, the implant, in particular the surface of the implant according to the invention comprises titanium or an alloy thereof. Such an implant surface is thus covered by a titanium oxide layer.

Accordingly, in an implant of the present invention, the oxide layer covering the surface of the implant is a metal oxide layer formed on the metallic surface of the implant.

In embodiments, the implant according to the invention may further be provided with a deposit on top of the oxide layer. Such a deposit may comprise a bone stimulating agent, such as lithium, strontium, calcium, magnesium or any other ion having a bone stimulating effect. Typically, the deposit comprises lithium ions.

As used herein the term “deposit” relates to a continuous or discontinuous film provided on top of the oxide layer. Such a deposit may

have any thickness and is not incorporated into the oxide layer, but is provided thereon.

Typically , the deposit is a salt precipitation comprising any one or a combination of ions selected from lithium, strontium, magnesium and calcium.

5 Usually, the deposit is a lithium salt precipitation, i.e. a lithium salt which is precipitated on top of the oxide layer of the implant surface.

Examples of suitable lithium salts are lithium hydroxide, lithium fluoride, lithium chloride, lithium sulphate, lithium nitrate, lithium carbonate. However, any lithium salt capable of being at least partly dissolved in the physiological fluid surrounding the implant may be used. Such salts are known to a person skilled in the art.

Upon implantation, a deposit comprising a salt precipitation dissolves easily and rapidly in the surrounding fluid such that the bone stimulating ions are released from the implant. An implant provided with such a deposit on its surface may be particularly beneficial in situations where an implant needs to integrate more rapidly, e.g. in bone of poor quality and quantity.

An advantage associated with an implant comprising a deposit of the above mentioned kind on its surface is that bone stimulating ions, e.g. lithium ions are easily and efficiently released into the physiological fluid surrounding the implant. Hence, a higher dose of bone stimulating ions, e.g. lithium ions may be released into the surrounding fluid.

Accordingly, the desired effect of lithium may be obtained both from ions present in the oxide in the oxide layer on the implant surface and ions released therefrom.

25 An implant according to the present invention suitably lacks a coating comprising a calcium phosphate compound. As outlined in the introduction, such implants are more prone to flake or break off from the implant surface, which may lead to an ultimate failure of the implant.

30 In embodiments of the invention, the implant surface may further comprise a micro-roughness having a root-mean-square roughness (R_q and/or S_q) of ≤ 250 nm (i.e. a micro-roughness comprising pores having a pore diameter of ≤ 1 μm and a pore depth of ≤ 500 nm) on at least a part of the implant surface. As used herein the term “nano- or micro-roughness” refers to a surface roughness comprising surface irregularities having
35 dimensions smaller than 1 μm

Such surface roughness is likely to give a larger contact and attachment area between the implant and the bone tissue, and provide a

better mechanical retention and strength between implant and bone. In alternative embodiments, the implant surface comprises fluorine and/or fluoride, such as 0.2-20 at%, and optionally also a micro-roughness having a root-mean-square roughness (R_q and/or S_q) of ≤ 250 nm, on at least a part of the implant surface.

Optionally, the surface of the implant according to the invention may comprise a macro-roughness. As used herein the term “macro-roughness” refers to a surface roughness comprising surface irregularities having dimensions greater than 1 μm .

It shall also be noted that the implant surface may be either threaded or unthreaded or it may be given other use dependent topographical features.

Furthermore, the present invention relates to a method for manufacturing a bone tissue implant having the characteristics outlined above, comprising the steps of:

- a) providing an implant having an implant surface;
- b) forming an oxide layer covering said implant surface;
- c) forming negatively charged ions on said oxide layer; and
- d) bringing said oxide layer into contact with lithium ions.

As previously mentioned, the implant may be a metallic implant, or it may be a non-metallic implant provided with a metallic surface. When non-metallic implants are used in the present invention, a metallic implant surface may be provided by any suitable technique known to those skilled in the art. For example, any suitable electrochemical treatment can be used.

An oxide layer covering the surface of the implant is preferably formed spontaneously, e.g. in contact with air. Such a layer is passive and inert, i.e. it is stable and prevents the underlying metallic surface from further reaction.

It is however possible to use any conventional oxidization techniques in the method above. Hence, the method is not limited to the spontaneous formation of an oxide layer. For instance, an oxide layer can be formed on a metallic implant surface by anodic oxidation of the implant in an electrolyte, such as an aqueous solution of an organic acid. An oxide layer can also be formed on a metallic implant surface by heating in air at, for instance, 150-1300°C. Moreover, an oxide layer can be formed on a metallic implant surface by precipitating the oxide on the implant surface from a suitable solution.

As already mentioned, an oxide layer covering said implant surface is preferably formed spontaneously, which is advantageous as no additional step of oxidation is actually required.

Referring to step c) in the method outlined above, negatively charged
5 ions may be formed on said oxide layer by subjecting the implant surface to an alkaline environment.

In contact with an aqueous solution the metal oxide, e.g. titanium oxide surface will disrupt the water molecule structure in its close vicinity, and, depending on the pH, become either positively or negatively charged. When
10 the surface is uncharged and no ions are adsorbed on the surface, the pH is called the point of zero charge pH_{PZC} . The pH_{PZC} for titanium oxide is between 5-7.

Hence, when the titanium oxide surface is surrounded by an aqueous, alkaline environment, e.g. an alkaline solution having a pH over 7, the surface
15 becomes slightly negatively charged due to the formation of surface bound, negatively charged hydroxide groups. Positively charged lithium ions, which may be present in a surrounding solution can thus be electrically attracted to the oxide surface (implant surface) and thereby become incorporated into at least part of the oxide layer, typically in the upper part of the oxide layer.
20 Preferably, the lithium ions are homogeneously distributed in the oxide layer.

An alkaline environment may be achieved locally on the surface of the oxide; i.e. negatively charged ions may be formed on the oxide layer by applying a potential which is more negative than -0.5 V; typically in the range of from -0.5 to -3.5 V. The application of such a potential will increase the
25 disruption of water molecules, generating the formation of hydrogen gas, and surface bound, negatively charged hydroxide groups on the implant surface.

Alternatively, an alkaline environment is achieved by subjecting the implant surface to an alkaline solution, e.g. by soaking the implant surface in an alkaline solution. Such an alkaline solution should have a pH higher than
30 7, e.g. higher than 10; and typically higher than 11. The soaking time may be less than 30 minutes, e.g. less than 20 minutes, typically between 10 and 15 minutes.

The implant surface is then brought into contact with positively charged lithium ions; e.g. by subjecting the implant surface to a solution comprising
35 lithium ions. The step of bringing said oxide layer into contact with lithium ions may be performed simultaneously with or after the step of forming negatively

charged ions on said oxide layer. Preferably the steps c) and d) of the method according to the invention are performed simultaneously.

For example, by applying a potential within the range of from -0.5 V to -3.5 V in a solution comprising lithium, negatively charged hydroxide groups will be formed, leading to an electrostatic interaction between surface bound hydroxide groups and lithium ions present in the solution. This electrostatic interaction results in that lithium ions are incorporated into the oxide layer. This is further described in Example 1.

The solution comprising lithium ions may be a solution comprising lithium hydroxide in a concentration of 5.3 M or less. The concentration of lithium hydroxide should not exceed 5.3 M, which represents the solubility product of lithium hydroxide. When the concentration of LiOH exceeds 5.3 M, salt crystals will be formed and precipitate on the surface of the oxide. In step c) of the method according to the invention, it is desired that the ions become incorporated into the oxide, and hence the concentration of LiOH should not exceed the solubility product.

A preferred concentration of lithium hydroxide is within the range of from 0.05 to 2 M.

The steps of forming negatively charged ions on the oxide surface and bringing said oxide layer into contact with lithium ions, thereby incorporating lithium ions into the oxide layer covering the surface of the implant is not limited to a specific method but may be achieved by any suitable method, or any combination of methods. For example, the implant surface may be anodized in an alkaline solution comprising lithium ions. Example 1 illustrates the incorporation of lithium ions by anodizing in lithium hydroxide.

By subjecting the implant surface to an anodization step, the thickness of the oxide layer will be affected. However, as the anodizing is preferably performed with a relatively low scan rate, e.g. below 6 mV/s until reaching 8V, the thickness of the oxide will not grow thicker than 100 nm.

Hence, lithium ions are incorporated into the oxide layer by means of the electrostatic interaction between negatively charged hydroxide groups formed on the implant surface and positively charged lithium ions present in a surrounding solution.

Optionally, the method according to the invention may comprise the
35 step of rinsing and/or cleaning said implant surface after step d). Furthermore,
the implant surface may be dried and sterilized after said rinsing step.

In embodiments, the method according to the invention further comprises the step of forming a deposit comprising a bone stimulating agent such as lithium, strontium, calcium, magnesium on top of said oxide layer, e.g. by precipitating a salt comprising the above mentioned ions on the surface of the implant; i.e. on the oxide layer covering said surface.

The salt may be any suitable salt of the ions above which is at least partly soluble in the physiological fluid surrounding the implant. The precipitation of a salt on the implant surface will form a continuous or a non-continuous film on the surface. The thickness of the deposit will depend on the amount of salt precipitated.

Such a salt deposit dissolves easily and rapidly in contact with the physiological fluid surrounding the implant such that the desired bone stimulating effect is achieved by the release of bone stimulating ions from the implant surface.

When the deposit is a lithium salt precipitation, the step of forming such a deposit may be achieved by modifying the above described methods for forming negatively charged ions on the surface of the oxide layer. For example, a potential more negative than -3.5 V can be applied. Such a negative potential gives rise to a significantly enhanced hydrogen gas development and an increased disruption of water molecules. Hence, an excess of negatively charged, surface bound hydroxide groups are formed at the oxide surface resulting in a deposit, i.e. a precipitate of lithium hydroxide on top of the oxide layer. See example 2 for further description.

Furthermore, by subjecting the implant surface to a solution comprising lithium hydroxide at a concentration above the solubility product of 5.3 M, a lithium salt deposit will be formed on the oxide surface (implant surface). This is also due to the excess of hydroxide groups in the surrounding.

However, the step of forming a deposit of e.g. a lithium salt is not limited to any specific method, but any method may be used. Neither is it limited to a specific lithium salt, but any salt which is at least partly soluble in the physiological fluid surrounding an implant may be used.

Furthermore, any method for forming a salt precipitation comprising any or a combination of the ions selected from lithium, strontium, magnesium and calcium can be used, e.g. the solution which comprises lithium may also comprise any or a combination of the above mentioned ions. In such cases, a small amount of these ions may also become incorporated into the oxide layer.

The step of forming a deposit may also be performed by a combination of the above mentioned methods.

It should however be noted that known methods for ion incorporation and deposit formation on an implant surface may also be used in the present

5 invention. Such methods include e.g.:

- plasma deposition, for instance using plasma source ion implantation or metal plasma immersion ion implantation,
- any electrochemical treatment, for instance voltametry in an electrolyte comprising lithium ions,
- 10 - treatment of the implant with an aqueous and/or non-aqueous solution comprising lithium ions, for instance by dipping said implant in said solution,
- treatment of the implant with a sol-gel technique,
- beam ion implantation,
- 15 - vacuum arc,
- filtered vacuum arc,
- metal vapour vacuum arc,
- ion plating,
- chemical vapour deposition,
- 20 - plasma assisted chemical vapour deposition,
- sputtering,
- laser ablation,
- providing a coating, such as a calcium phosphate-containing coating or a silane coating, on the implant surface, in or to which lithium ions
- 25 can be incorporated or attached,

any combination of these methods or the like. The method according to the invention may further comprise the step of creating a micro roughness on the implant surface.

Before, simultaneously with and/or after the provision of lithium ions or
30 a salt thereof on the implant surface, a nano- and/or micro-roughness can be optionally provided on the implant surface using, for instance, mild etching, micro-fabrication, anodization, flame spraying, electrochemical treatment, laser, spark erosion, or any other suitable method of surface modification. Reference can be made to WO 04/008983 and WO 04/008984, wherein
35 suitable methods for obtaining such an implant surface are disclosed. It is however preferred that the nano- and/or micro-roughness is provided after step a) in the method according to the invention.

The method of the invention may also comprise the step of applying fluorine to the implant surface. Reference can be made to WO 04/008983, wherein suitable methods for obtaining such an implant surface are disclosed.

A suitable method, according to WO 04/008983, for providing fluorine and/or fluoride and a micro-roughness having a root-mean-square roughness (R_q and/or S_q) of ≤ 250 nm on at least a part of an implant surface is by treatment of the implant with an aqueous solution of hydrofluoric acid having a concentration of less than 0.5 M, such as 0.1 M, for an etching period of up to 180 sec, such as 60 s, at room temperature (see WO 04/008983 for more information).

In addition, a macro-roughness can be optionally provided on the implant surface prior to providing lithium ions or a salt thereof, and prior to optionally providing the micro-roughness, thereon. A macro-roughness can, for instance, be provided by blasting, e.g. with titanium dioxide particles, etching, micro-fabrication, anodization, flame spraying, any electrochemical treatment, laser, spark erosion, machining, knurling, or any other suitable method of surface modification.

It shall also be noted that the implant surface may be either threaded or unthreaded or be given other use dependent topographical features.

20 The method for manufacturing a bone tissue implant according to the invention is not limited to the incorporation of lithium ions, but may be applied to incorporate positively charged ions into the implant surface in general. Hence, such a method involves the steps of:

- 25
- a) providing an implant having an implant surface;
 - b) forming an oxide layer covering said implant surface;
 - c) forming negatively charged ions on said oxide layer; and
 - d) bringing said oxide layer into contact with positively charged ions.

The present invention further relates to a blasting powder, which comprises a metal oxide, wherein the metal oxide comprises lithium ions.

30 The metal oxide may be a metal oxide selected from the group consisting of titanium oxide, zirconium oxide, hafnium oxide, tantalum oxide, and niobium oxide. Preferably the blasting powder comprises titanium oxide into which lithium ions are incorporated.

It is also possible to simply use lithium oxide as the blasting powder of
35 the present invention.

It may be possible to use said blasting powder in the method according to the invention to further enhance the incorporation of lithium ions into the

oxide layer covering the implant surface. However, a blasting powder may be used by itself to incorporate lithium ions into any oxide layer provided on any implant surface.

The present invention also relates to a method for locally increasing bone formation. Such a method comprises administering a composition comprising lithium ions or a salt thereof and a pharmaceutically acceptable carrier to a person in need thereof. Preferably, the composition comprising lithium ions or a salt thereof and a pharmaceutically acceptable carrier is administered at an implantation site upon implantation of an implant into bone tissue at said implantation site before, simultaneously with and/or after said implant is placed in a cavity in the bone tissue at said site.

The composition comprising lithium ions, or a salt thereof, can be administered in and/or nearby said cavity in the bone tissue.

Examples of suitable pharmaceutically acceptable carriers for use in said composition are a physiological saline solution; disintegrated autologous bone; a demineralised bone matrix; liposomes; nano- or microparticles of biodegradable polymer(s), such as polylactic acid (PLA) and polyglycolic acid (PGA); hyaluronic acid; collagen; chondroitin sulfate; a hydrogel, such as alginate; chitosan; a scaffold of polyester and tricalcium phosphate; and the like.

A specific example of a suitable carrier is PepGen P-15 PUTTY™, which are particles of hydroxyapatite enhanced with P-15, a synthetic peptide that mimics the cell-binding region of Type-I collagen, suspended in sodium hyaluronate.

25 The composition according to the invention can either be an immediate release, a delayed release or a controlled release formulation.

The invention also relates to the use of lithium ions, or a salt thereof, for manufacturing a pharmaceutical composition (as disclosed above) for locally increasing bone formation.

30 The composition may be locally administered at an implantation site upon implantation of an implant into bone tissue at said site.

According to the present inventors, local administration of lithium ions or a salt thereof directly into the bone tissue is preferred over systemic administration. Foreign agents often have a variety of effects on the human body, which are both known and unknown. Local administration of lithium or a salt thereof in bone tissue is beneficial as the bone stimulating effect will be achieved, while side effects are avoided.

5 While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent for one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Examples

15

Titanium samples were reduced by potential step technique in 0.1M LiNO_3 , pH 5-6. A potential step of -3V (all potentials are referred to a double junction $\text{Ag}/\text{AgCl}/\text{KCl}$ reference electrode, 197 mV against SHE) was applied over the sample for 5 minutes resulting in a continuous hydrogen evolution. After the reduction process the samples were rinsed in MQ water in an ultrasonic bath for 2 minutes before drying and sterilization. The presence and distribution of lithium was identified by Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) (figure 1).

25

The presence and distribution of lithium was identified by TOF-SIMS (figure 3).

Example 2: Release of lithium from a titanium implant surface

Titanium samples were reduced by potential step technique in 0.1M LiNO₃, pH 5-6. A potential step more negative than -4V (all potentials are referred to a double junction Ag/AgCl/KCl reference electrode, 197 mV against SHE) was applied over the sample for 5 minutes resulting in vigorous hydrogen evolution. After the reduction process the samples were rinsed in water and dried.

The release of lithium was identified by ISE (Ion Selective Electrode) and ICP (Inductively Coupled Plasma). Four coin shaped samples, prepared according to the above, were placed in a beaker with 15 ml MQ water. The water was acidified to pH 4 using 20mM HNO₃ and thereafter left for 90 minutes with moderate agitation before analysis. The solution was analysed using ISE and ICP, both analyses giving the same result of 0.4 µg lithium /ml sample.

Reference example 1: Culturing of MG-63 cells

MG-63 is a human cell line (ATCC No CRL-1427, U.S.) conventionally used in the art for in vitro studies of osteoblasts. MG-63 originates from a human osteosarcoma and exhibits numerous trait characteristics of human osteoblasts, such as alkaline phosphatase (ALP), and osteoprotegerin (OPG).

In this study, MG-63 cells were obtained from frozen cells in second passage and further cultured in Dulbecco's modified Eagle's medium (DMEM) containing FCS 5%, PEST 1% , Gibco, UK) in 37°C in an atmosphere of 5% CO₂ and 100% humidity. When the cells reached confluence they were subcultured by the use of 0.05% Trypsin-EDTA (Gibco, UK) until the amount of cells were obtained.

Cell viability was high in all experiments (>98%) and was checked by the use of trypan blue where the uptake of the stain by dead cells was checked in a Bürkerchamber in a light microscope (LM).

Reference example 2: Proliferation of MG-63 cells and production of alkaline phosphatase (ALP)

In order to study the effect of lithium on the proliferation of MG-63 cells and the production of alkaline phosphatase (ALP), MG-63 cells prepared in reference example 1 were subcultured into 24 well plates at a plating density of 10 000 cells/cm², in total 20 000 cells/well. Sterile filtered LiNO₃ at final concentrations of 10mM, 5mM, 3mM, and 1mM respectively (pH 5.2) were

added to the respective wells of the plate. Untreated cells were used as controls. Cells were cultured for 3, 7 and 14 days at a temperature of 37°C in an atmosphere of 5% CO₂ and 100% humidity.

The total number of cells in each well ($\times 10^5$) after each time period
5 was determined by the NucleoCassette method by the NucleoCounter
(ChemoMetec A/S Denmark).

The number of cells was investigated by lysis of the cells in “Reagent A” having a pH of 1.25 following stabilization by “Reagent B”. In the NucleoCassette, propidium iodide was incorporated which targets the amount of released DNA. The cassette was placed in the NucleoCounter and the amount of measured fluorochrome corresponded to the amount of DNA. The instructions from the manufacturer were followed (Chemometec A/S, Denmark).

At harvest, the cell culture medium was analysed with respect to the ALP content. The intracellular ALP was measured by cell lysis according to the instructions of the manufacturer (SenzoLyte™ pNPP Alkaline Phosphatase Assay Kit Colorimetric BioSite, Sweden). The absorption was set at 405 nm by an automatic platereader (Vmax, Molecular Device, UK). By comparing the optical density of the samples with the standard provided with the kit, the ALP concentrations could be determined. The instructions from the manufacturer were followed (BioSite, Sweden).

Referring to figure 4, an increased proliferation of MG-63 cells was achieved at all the three time points with 5, 3 and 1 mM lithium. The highest detected cell number was observed after 7 days.

25 The production of ALP was initially slow with all lithium concentrations compared to control (unstimulated cells). A small increase was detected after 7 days compared to the control. However, increased ALP levels were detected after 14 days with all lithium concentrations; 1 mM, 3 mM, 5 mM and 10 mM. The highest amount of ALP was observed with 1 mM lithium after 30 14 days. The results are illustrated in figure 5.

Reference example 3: Preparation of reference surfaces

Titanium samples having the shape of a coin were cleaned, and then immersed in an 1 M aqueous solution of oxalic acid and left at 80°C for 30 minutes under vigorous agitation. After 30 minutes the samples were removed from the oxalic acid solution and rinsed in water followed by rinsing

Some of the samples were subjected to a secondary hydrofluoric acid treatment. Approximately 10 minutes after rinsing, the samples were immersed in 0.1 M aqueous solution of HF at room temperature and agitation until the start of active dissolution, followed by an additional active treatment time of 40 s. Next, the samples were removed from the HF solution and rinsed in water followed by rinsing in water in an ultrasonic bath for 2 minutes. The samples were dried in air at room temperature for about 60 minutes before sterilization. The resulting surface is referred to as “reference surface 2”

15 Sterilized (β -radiation) Ti coins with reference surfaces 1 and 2,
respectively were placed in 24 well plates. MG-63 cells were subcultured onto
the coins in the 24 well plates at a plating density of 10 000 cells/ cm², in total
20 000 cells/well. Sterile filtered LiNO₃ at final concentrations of 5mM and
1mM (pH 5.2) were added to the respective wells. Untreated cells were used
as controls. Cells were cultured for 7 days at a temperature of 37°C in an
20 atmosphere of 5% CO₂ and 100% humidity.

The reference surface 1 comprising lithium in a concentration of 1 mM induced an increased proliferation of MG-63 cells after 7 days of culture. The reference surface 2 comprising lithium in a concentration of 1 mM induced a high cell proliferation after 7 days of cell culture compared to the unstimulated reference surface 2. The final concentration of 5 mM lithium induced a modest increased proliferation. The results are illustrated in figure 6.

30 The production of ALP on reference surface 1 comprising a concentration of 1mM lithium was compared with unstimulated reference surface 1 comprising no lithium.

As is illustrated in figure 7, the ALP production increased with 1 mM lithium after 7 days of cell culture.

Titanium samples having (i) reference surface 1 (ii) reference surface 2, and (iii) reference surface 1 + lithium were prepared for Scanning Electron

The figures 8, 9, and 10 illustrate the morphology of the respective surface after 36h. The reference surface 1 comprising lithium has a larger amount of cells in proliferative stage (fig. 10) (round, non-spread cells) as well as a large amount of adhesive cells compared to cells on the reference surface 1 and 2 comprising no lithium. On both the reference surface 1 (figure 8) and reference surface 2 (figure 9) the cells are thinner, flatter and more spread out.

The morphology of the cells cultured on the reference surface 1 comprising lithium shows that the cells are well spread out, indicating high activity, proliferate, and form matrix and pseudopodia on the surfaces. This indicates that such surfaces are osteoconductive as well as osteoinductive and in favour for cell adhesion, proliferation and matrix formation.

25 Sterilized (β radiation) Ti coins with reference surfaces 1 and 2 were compared with Ti coins with reference surface 1 comprising lithium, calcium and magnesium, respectively, and the coins were placed in 24 well plates. MG-63 cells were subcultured onto the coins in the 24 well plates at a plating density of 10 000 cells/ cm², in total 20 000 cells/well. Cells were cultured for 7 days at a temperature of 37°C in an atmosphere of 5% CO₂ and 100% humidity.

The number of cells was investigated by lysis of the cells in “Reagent A” having a pH of 1.25 following stabilization by “Reagent B”. In the NucleoCassette, propidium iodide was incorporated which targets the amount of released DNA. The cassette was placed in the NucleoCounter and the amount of measured fluorochrome corresponded to the amount of DNA. The

instructions from the manufacturer were followed (Chemometec A/S, Denmark).

Referring to figure 11, the proliferation with the reference surface 1 comprising lithium showed the highest MG-63 cell proliferation after 7 days of cell culture. The cell proliferation was markedly higher than the reference surfaces comprising calcium and magnesium.

Comparative example 2: Production of osteoprotegerin on reference surface 1

The production of osteoprotegerin (OPG) was compared between Ti coins with reference surface 1 comprising lithium, calcium and magnesium, respectively.

The amount OPG was determined by DuoSet ELISA human OPG/TNFRSF11B (R&D Systems,UK). The supernatant from each well was centrifuged free of cells (5 min in 400g) and further used for investigation. Sample OPG concentrations were determined by correlating the amounts to the standard curve provided by the manufacturer. The sensitivity of the test (MDD, minimum detectable dose) was 50 pg/ml. The instructions from the manufacturer were followed (R&D Systems,UK).

As is illustrated in figure 12, the highest production of OPG was observed with reference surface 1 comprising lithium after 7 and 14 days of cell culture.

Comparative Example 3: Bone tissue response *in vivo*

Integration of implants according to the invention was tested in a rabbit model. The objective was to qualitatively and quantitatively study the *in vivo* bone tissue response of implant surface modifications according to the invention compared to a commercially available control implant.

30 Implants for removal torque study

Torque fixtures (square headed removal torque design, 3.5 x 8.2 mm) with reference surface 1 comprising lithium were compared with control torque fixtures (3.5x8.2 mm) with the commercially available Osseospeed™ surface.

35

Implant insertion

25

Twelve mature male New Zealand white rabbits were scheduled for surgery. Two rabbits died during initial anaesthesia (#9, 10). The surgery went uneventful. Low speed drilling (1500 rpm for drilling the holes and 20 rpm for implant insertion) was done with continuous NaCl cooling. The first drill
5 was a small round burr and thus used as a marker for the coming larger spiral drills (altogether 6 drills having diameters in the range of from 1.2 to 3.35 mm).

Three implants ("square headed removal torque design"; 3.5 x 8.2 mm) were inserted in each tuberositas tibia. The tibia implants were scheduled for
10 removal torque tests.

Removal torque results

After six weeks the study was terminated and the rabbits were sacrificed. The implants and surrounding tissue were examined. The tibia rtq
15 implants were easy to locate and all of them showed signs of periosteal bone tissue up-growth. The biomechanical test of the implant-bone interface was performed with the removal torque test (RTQ). The RTQ instrument is an electronic equipment (Detektor AB, Göteborg, Sweden) involving a strain gauge transducer used for testing the implant stability (the peak loosening
20 torque in Ncm) in the bone bed and can thus be regarded as a three dimensional test roughly reflecting the interfacial shear strength between bone tissue and the implant (Johansson C. B. , Albrektsson T. *Clin Oral implants Res* 1991; 2:24-9). A linear increasing torque was applied on the same axis of the implant until failure of integration was obtained and the peak
25 value was noted.

As is illustrated in figure 13, the removal torque value for the implant comprising lithium according to the invention was improved compared to the commercially available Osseospeed™ surface.

30

CLAIMS

1. An implant for implantation into bone tissue having a surface; said surface being covered by an oxide layer c h a r a c t e r i z e d in that said
5 oxide layer comprises lithium ions.

2. An implant according to claim 1, wherein said implant is a metallic implant.

10 3. An implant according to claim 2, wherein said metallic implant comprises titanium or an alloy of titanium.

4. An implant according to claim 1, wherein said implant is a non-metallic implant; said surface being an applied metallic implant layer.
15

5. An implant according to claim 4, wherein said metallic implant layer comprises titanium or an alloy of titanium.

6. An implant according to any one of the preceding claims, wherein
20 said oxide layer has a thickness within the range of from 2 to 100 nm.

7. An implant according to claim 6, wherein said oxide layer has a thickness within the range of from 2 to 18 nm.

25 8. An implant according to claim 6 or 7, wherein said oxide layer has a thickness within the range of from 2 to 6 nm.

9. An implant according to any one of the preceding claims, wherein said oxide layer is a metal oxide layer formed from said surface of said
30 implant.

10. An implant according to any one of the preceding claims further comprising a deposit on top of said oxide layer, wherein said deposit comprises a bone stimulating agent.
35

11. An implant according to claim 10, wherein said bone stimulating agent is selected from the group consisting of lithium, strontium, magnesium and calcium or a combination thereof.

5 12. An implant according to claim 10 or 11, wherein said deposit is a salt precipitation comprising any one or a combination of ions selected from lithium, strontium, magnesium and calcium.

10 13. An implant according to claim 12, wherein said salt precipitation is a lithium salt precipitation.

14. An implant according to any one of the preceding claims, wherein said implant lacks a coating comprising a calcium phosphate compound.

15 15. An implant according to any one of the preceding claims, wherein
said surface of said implant comprises a micro roughness.

16. An implant according to any one of the preceding claims, wherein said surface of said implant comprises fluorine.

20

17. A method for manufacturing a bone tissue implant having an implant surface covered by an oxide layer comprising lithium ions, said method comprising the steps of:

25 a) providing an implant having an implant surface;
b) forming an oxide layer covering said implant surface;
c) forming negatively charged ions on said oxide layer; and
d) bringing said oxide layer into contact with lithium ions.

18. A method according to claim 17, wherein said oxide layer is formed
30 spontaneously.

19. A method according to claim 17 or 18, wherein said negatively charged ions on said oxide layer are formed by subjecting said implant surface to an alkaline environment.

35

20. A method according to claim 19, wherein said alkaline environment is formed by subjecting said implant surface to an alkaline solution.

21. A method according to any one of the claims 17-19, wherein said negatively charged ions on said oxide layer are formed by applying a potential within the range of -0.5 V to -3.5 V.

5

22. A method according to any one of the claims 17-21, wherein said step c and said step d are performed simultaneously.

23. A method according to any one of the claims 17-22, wherein said
oxide layer is brought into contact with lithium ions by subjecting said oxide
layer to a solution comprising lithium ions.

24. A method according to claim 23, wherein said solution comprises lithium hydroxide.

15

25. A method according to claim 24, wherein said solution comprises lithium hydroxide in a concentration of 5.3 M or less.

26. A method according to claim 25, wherein said solution comprises
20 lithium hydroxide in a concentration in the range of from 0.05 to 2 M.

20

27. A method according to any one of the claims 17-26, further comprising the step of forming a deposit comprising a bone stimulating agent on top of said oxide layer.

25

28. A method according to claim 27, wherein said bone stimulating agent is selected from the group consisting of lithium, strontium, calcium and magnesium or a combination thereof.

30 29. A method according to claim 27 or 28, wherein said deposit is
formed by precipitating a salt comprising any one or a combination of the ions
selected from lithium, strontium, calcium and magnesium on said oxide layer.

30

30. A method according to claim 29, wherein said salt is lithium
35 hydroxide.

35

29

31. A method according to claim 29 or 30, wherein said deposit is formed by applying a potential which is more negative than -3.5 V.

5 32. A method according to claim 29 or 30, wherein said deposit is formed by subjecting said implant surface to an alkaline solution comprising lithium hydroxide in a concentration of more than 5.3 M.

10 33. A method according to any one of the claims 17-32, further comprising the step of creating a micro roughness on said surface of said implant after step a).

34. A method according to any one of the claims 17-33, further comprising the step of applying fluorine to said surface of said implant.

15 35. A blasting powder comprising a metal oxide, wherein said metal oxide comprises lithium ions.

20 36. A blasting powder according to claim 35, wherein said metal oxide is titanium dioxide.

37. A blasting powder according to claim 35, wherein said metal oxide is lithium oxide.

25 38. A method for locally increasing bone formation by administering a composition comprising lithium ions or a salt thereof and a pharmaceutically acceptable carrier to a person in need thereof.

30 39. A method according to claim 38, wherein said composition is administered at an implantation site upon implantation of an implant into bone tissue at said implantation site before, simultaneously with and/or after said implant is placed in a cavity in the bone tissue at said site.

40. Use of lithium ions or a salt thereof for manufacturing a pharmaceutical composition for locally increasing bone formation.

35

41. Use according to claim 40, wherein said composition is locally administered at an implantation site upon implantation of an implant into bone tissue at said site.

- 5 42. A kit for implantation of an implant into bone tissue comprising an implant c h a r a c t e r i z e d in that said kit further comprises a composition comprising lithium ions or a salt thereof and a pharmaceutically acceptable carrier.

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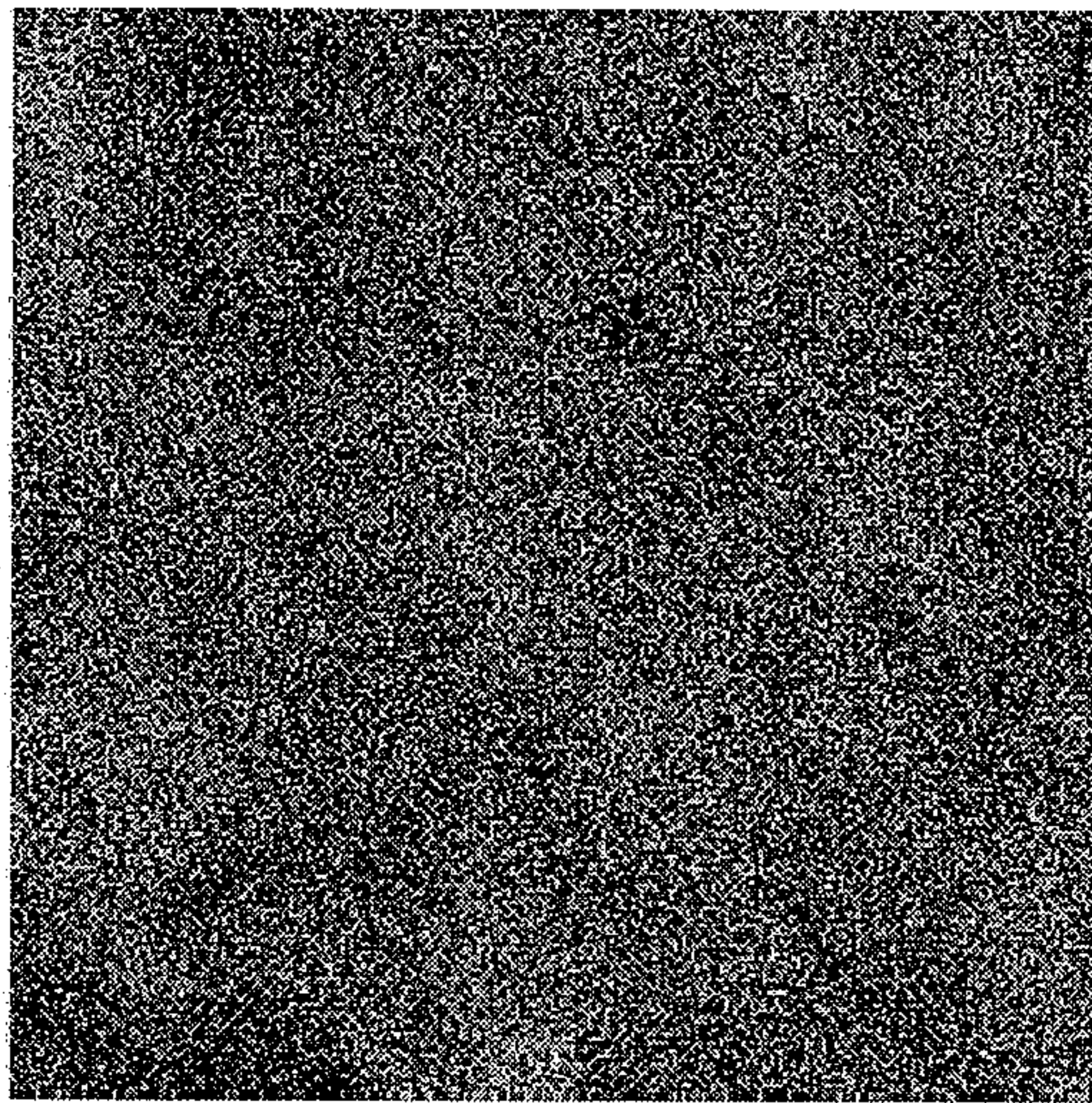
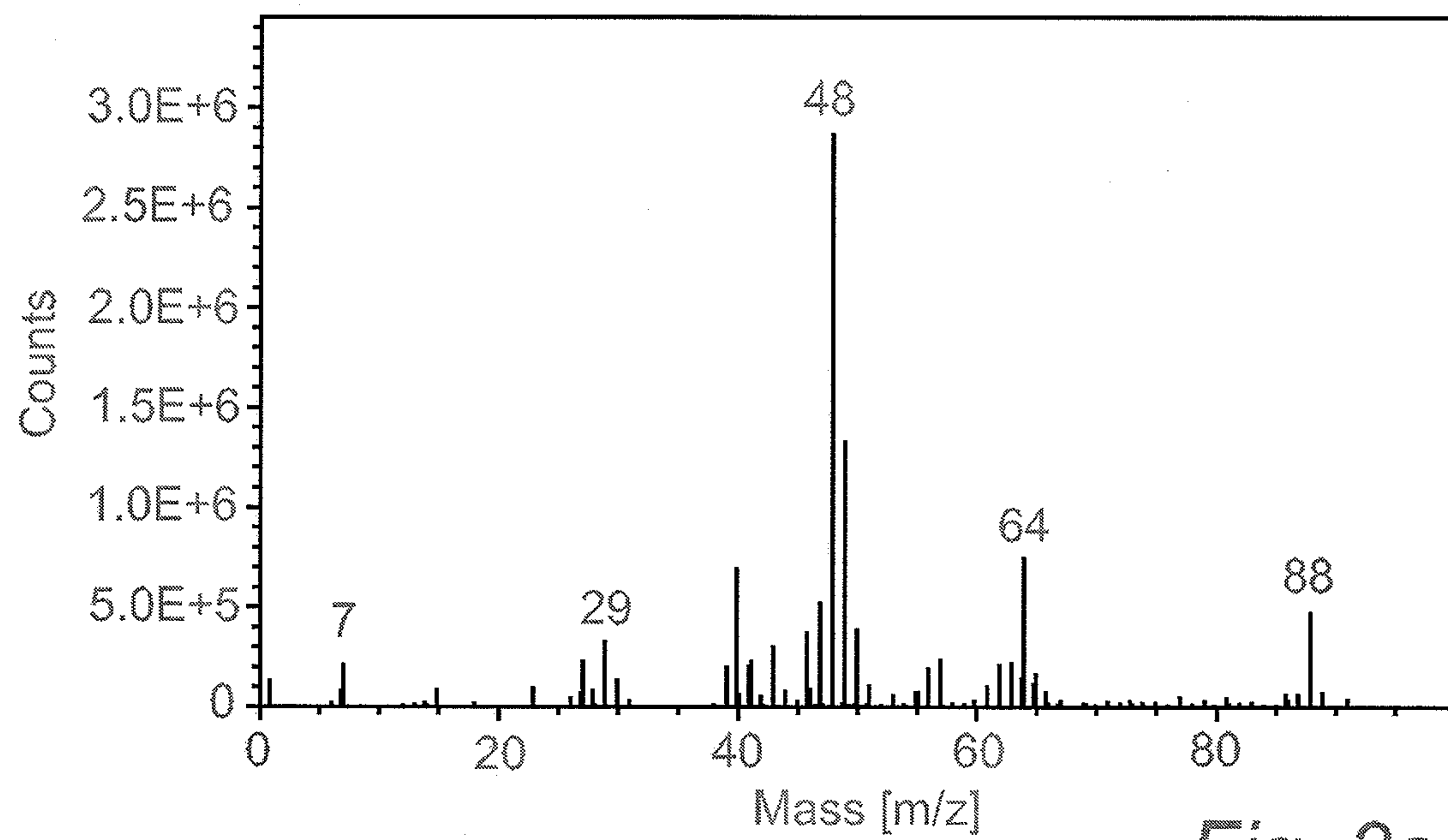
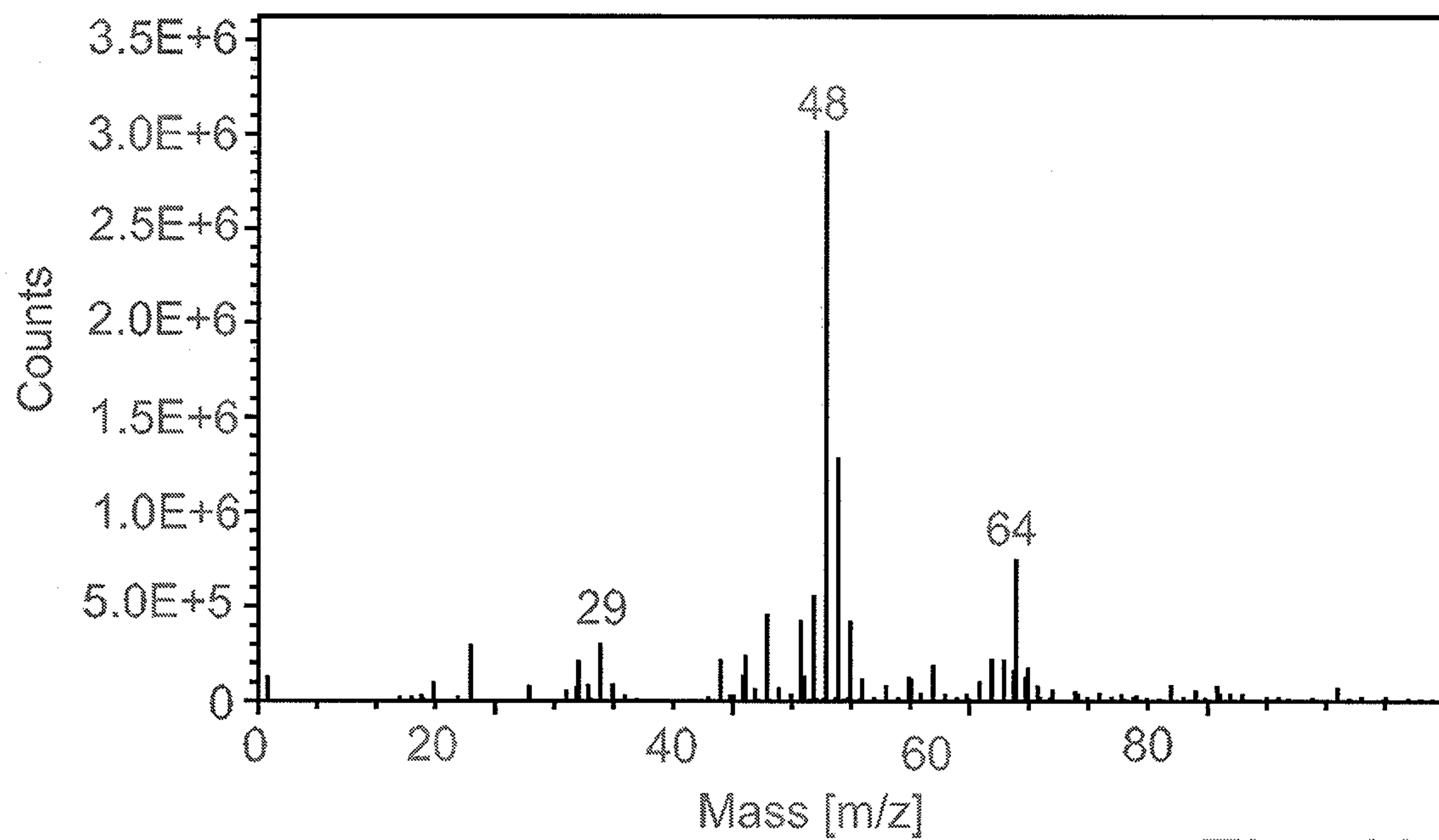


Fig. 1

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*Fig. 2a**Fig. 2b*

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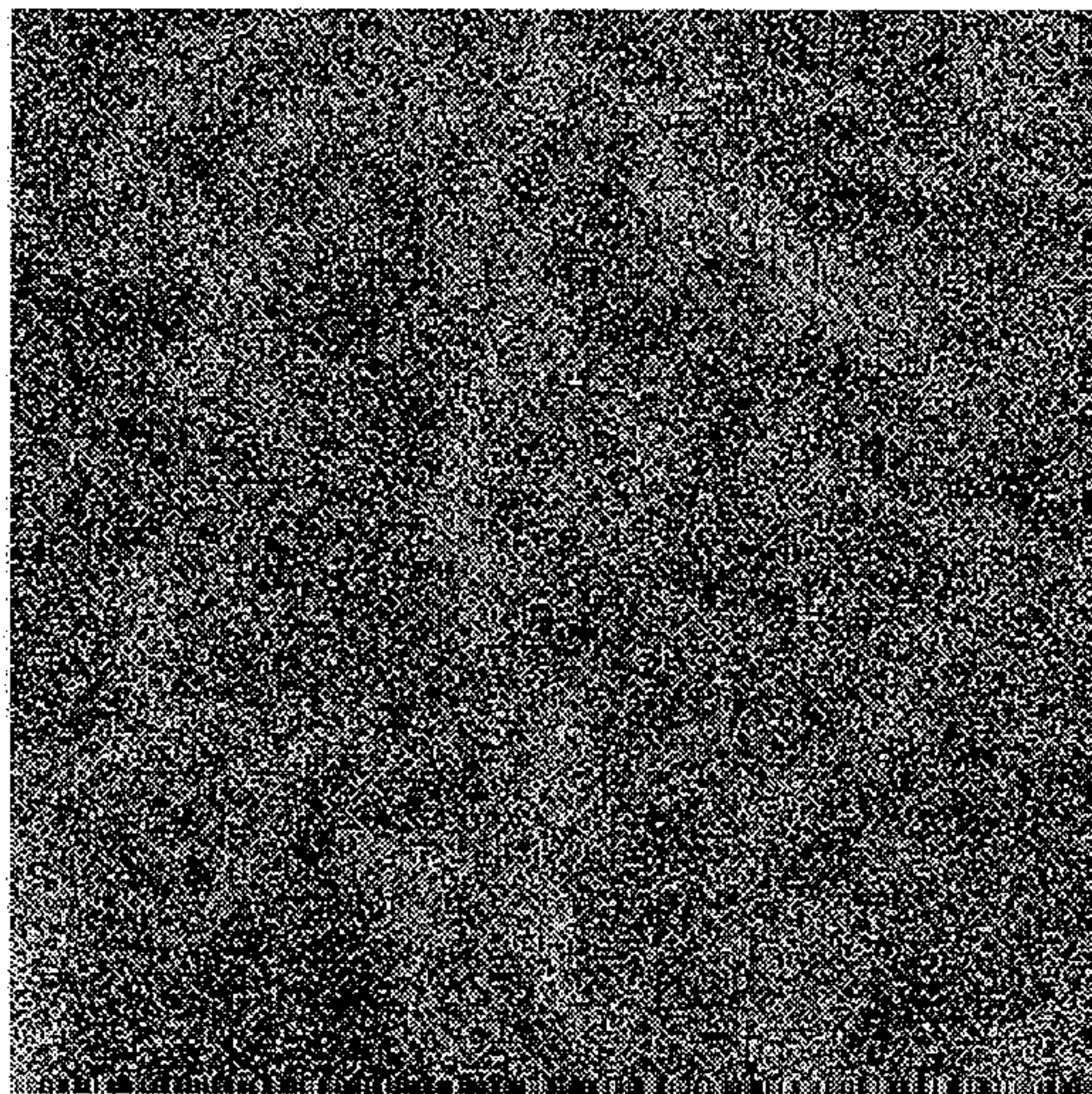
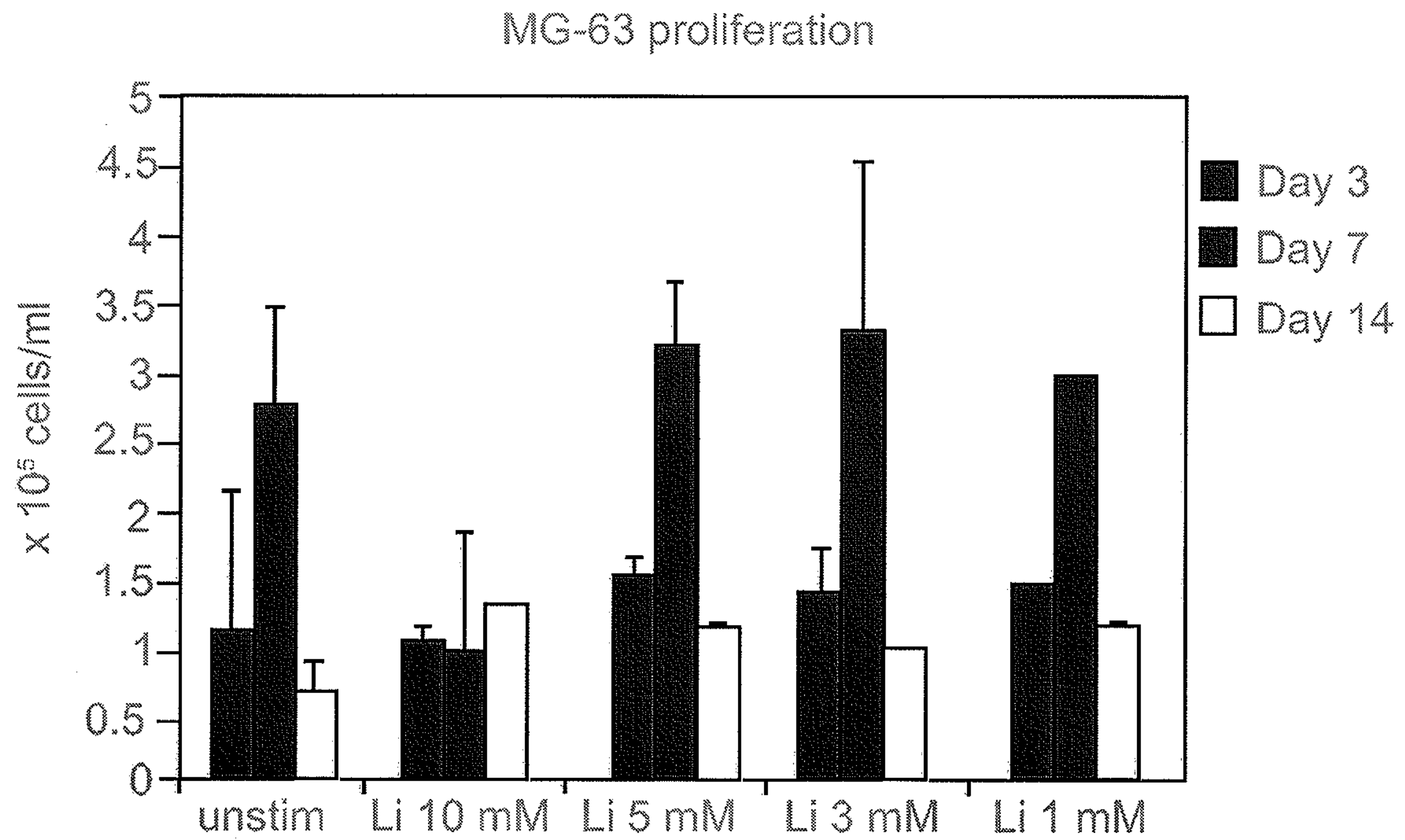
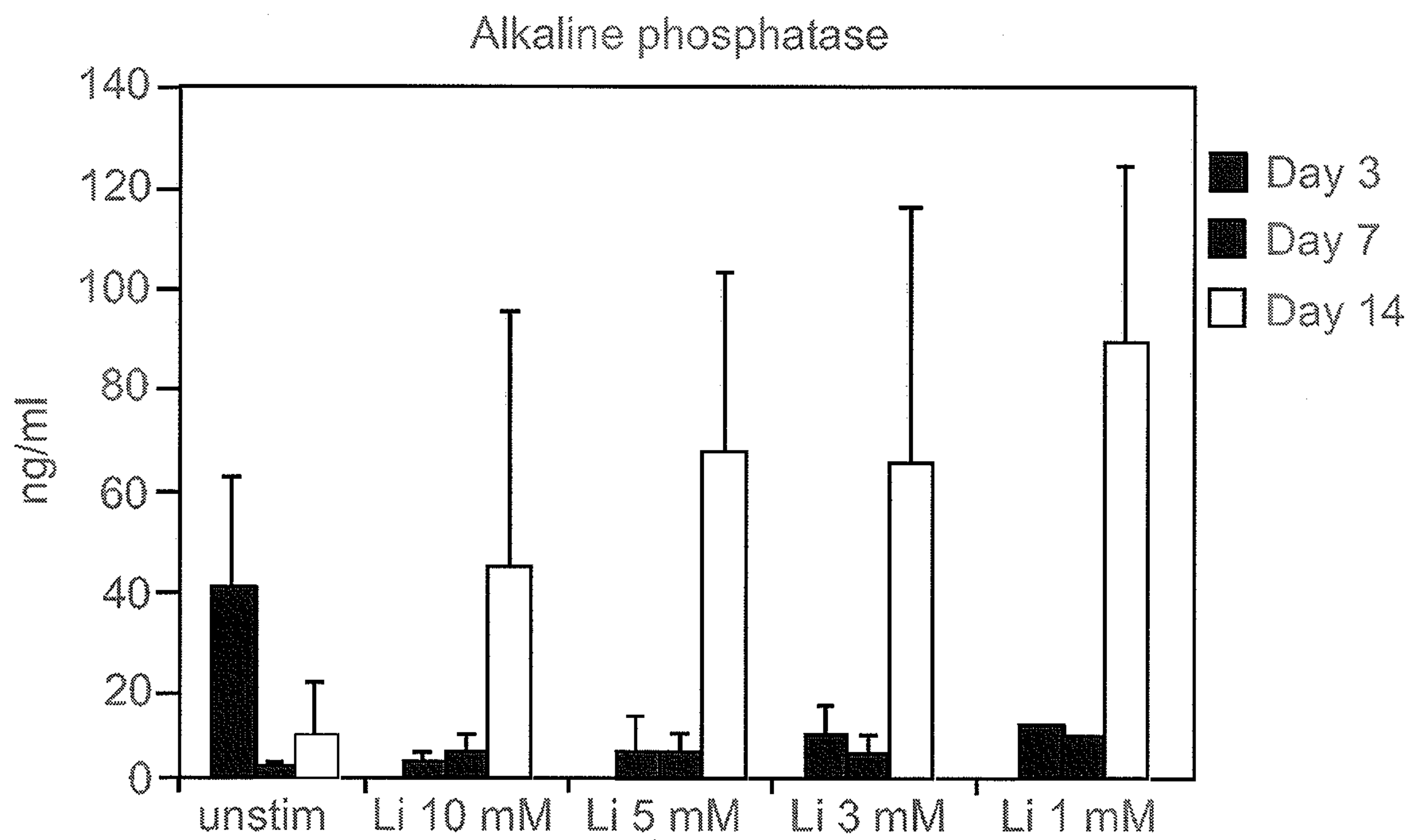
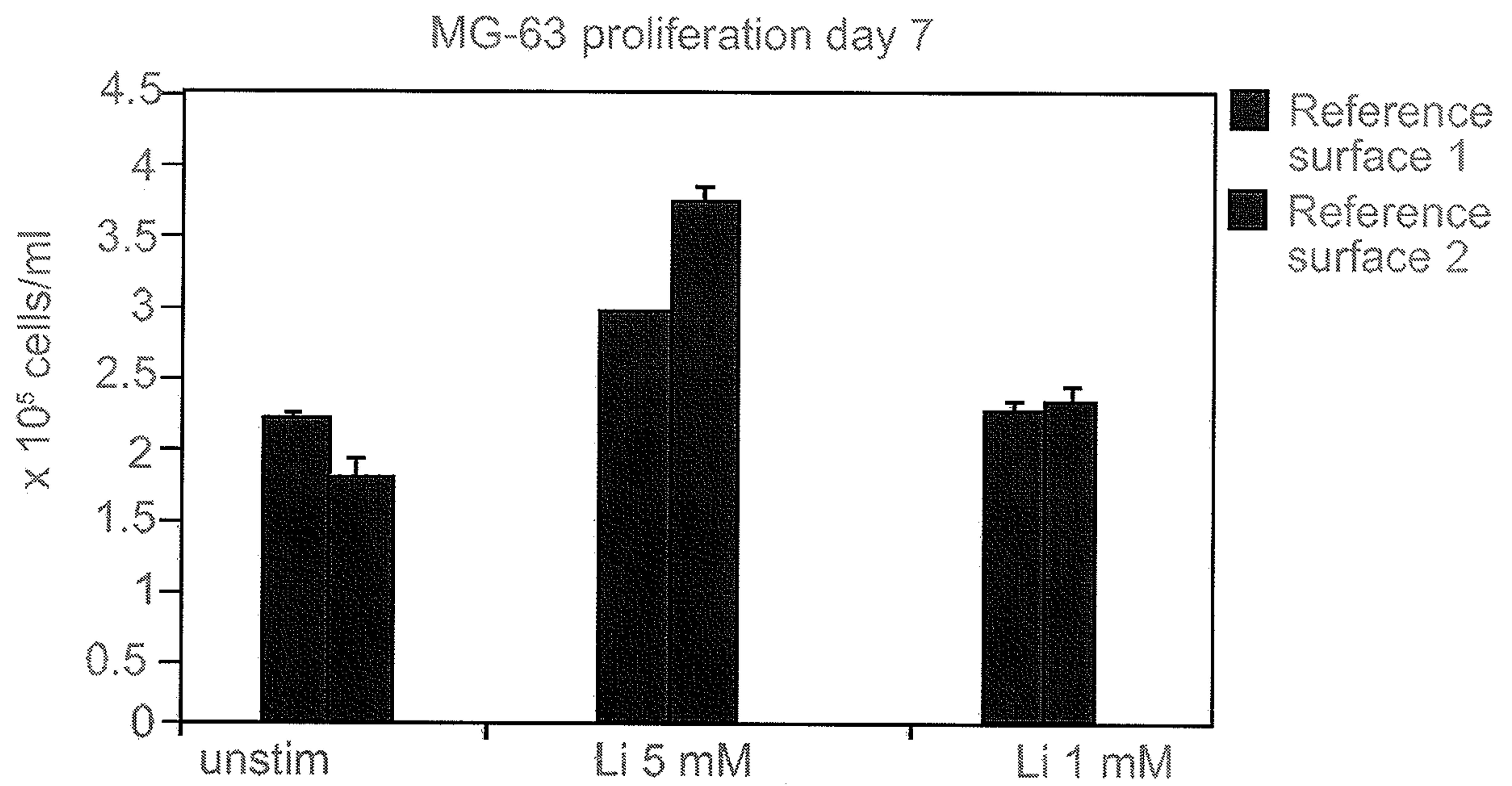
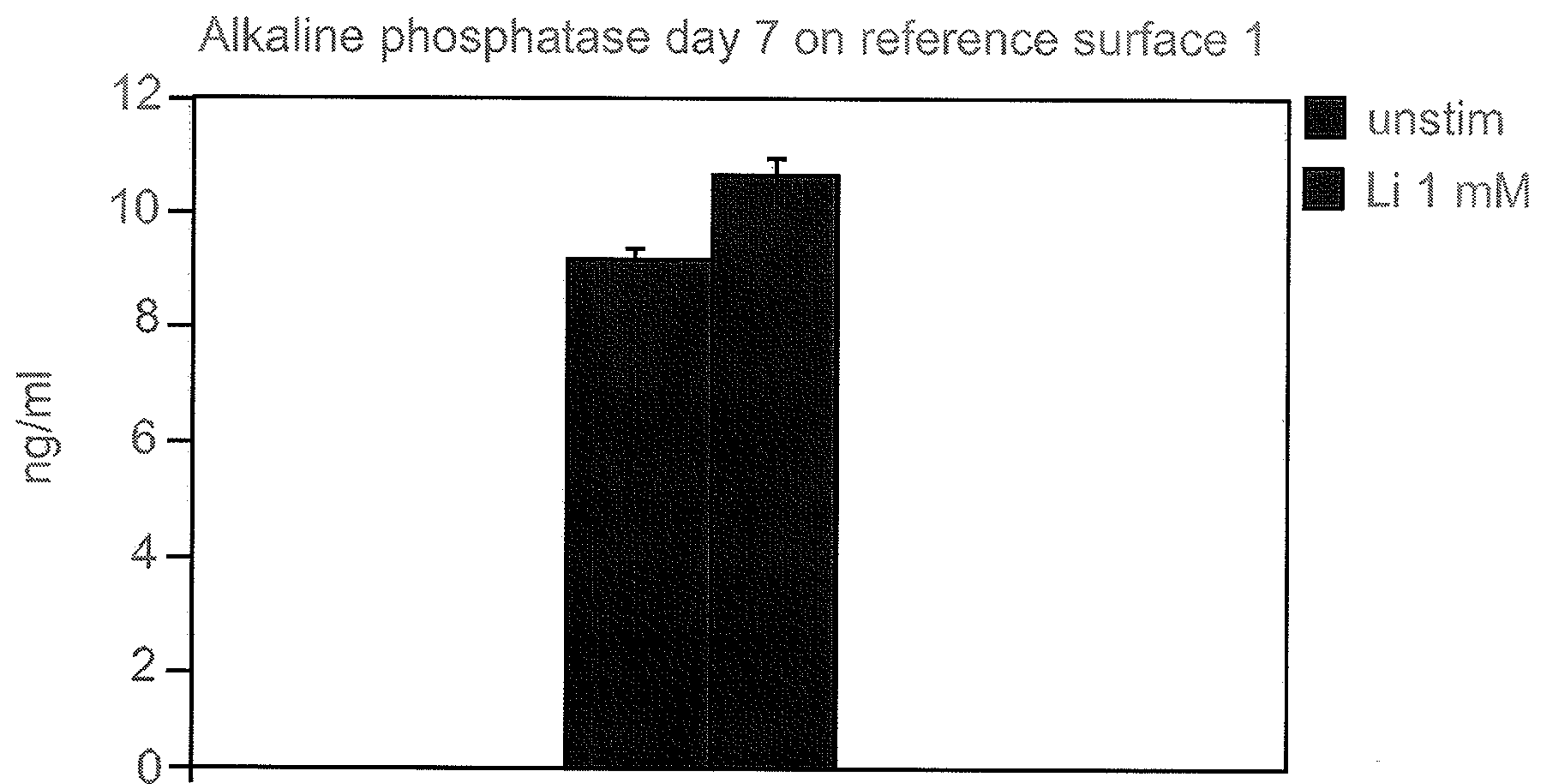


Fig. 3

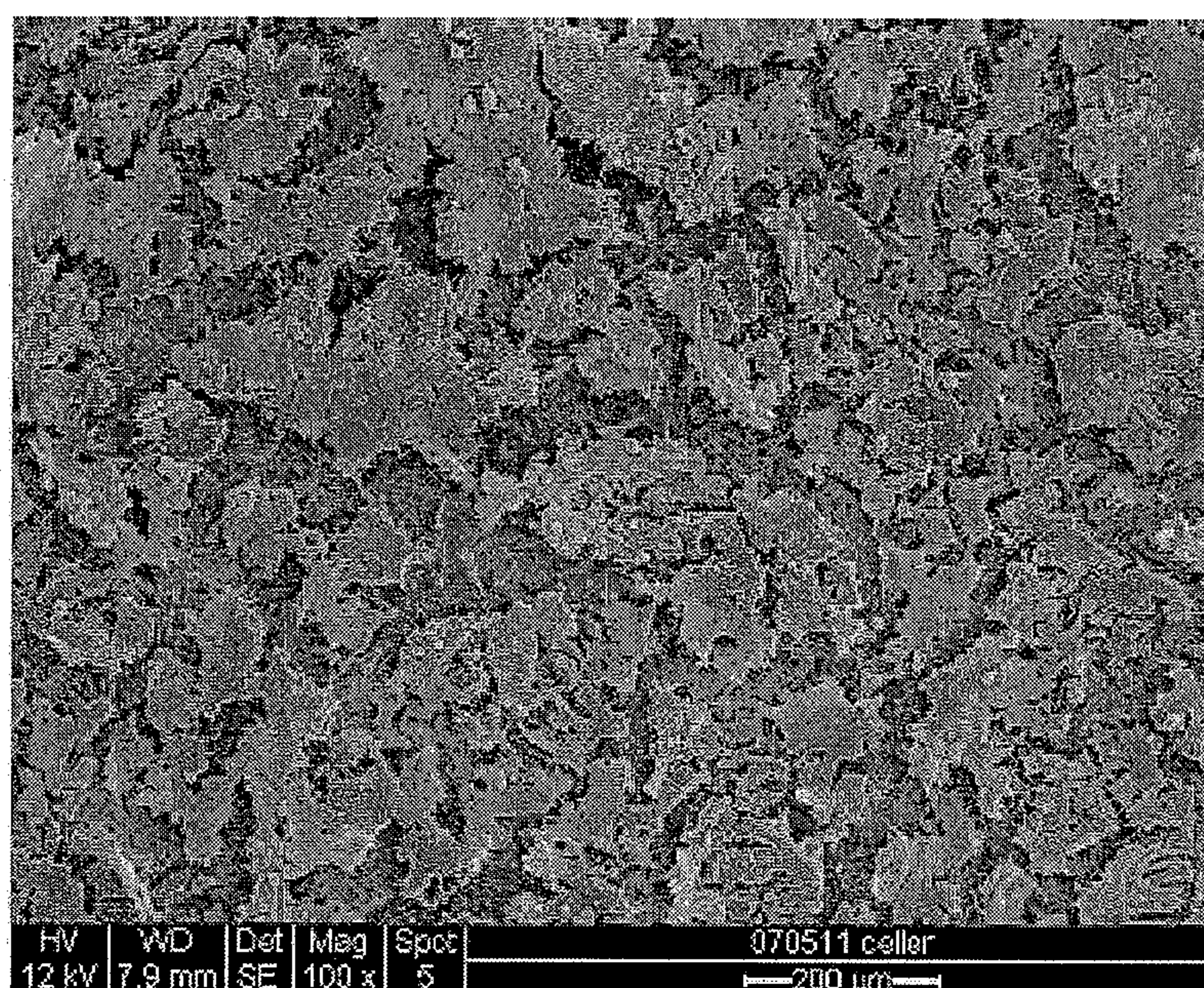
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*Fig. 4**Fig. 5*

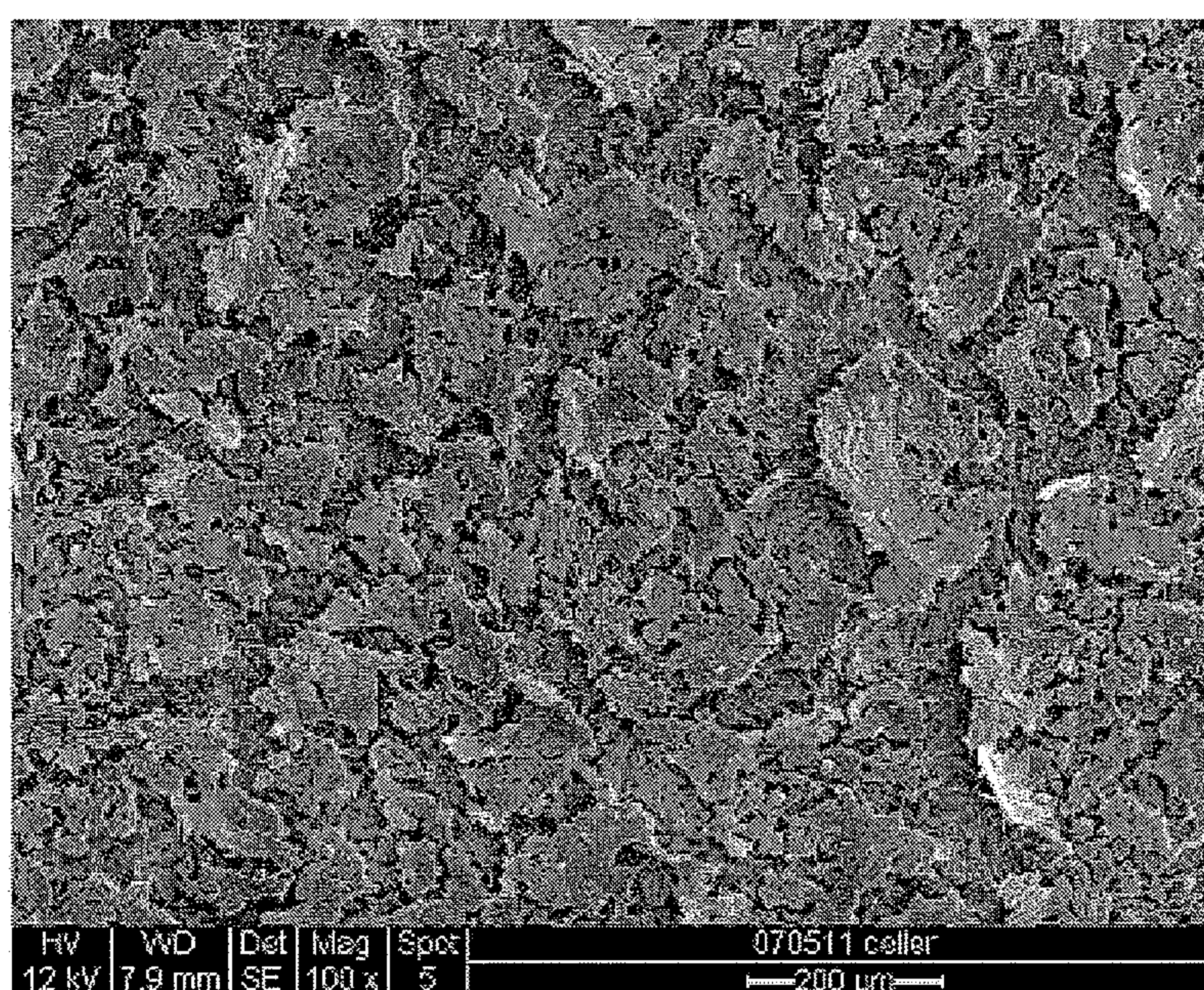
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*Fig. 6**Fig. 7*

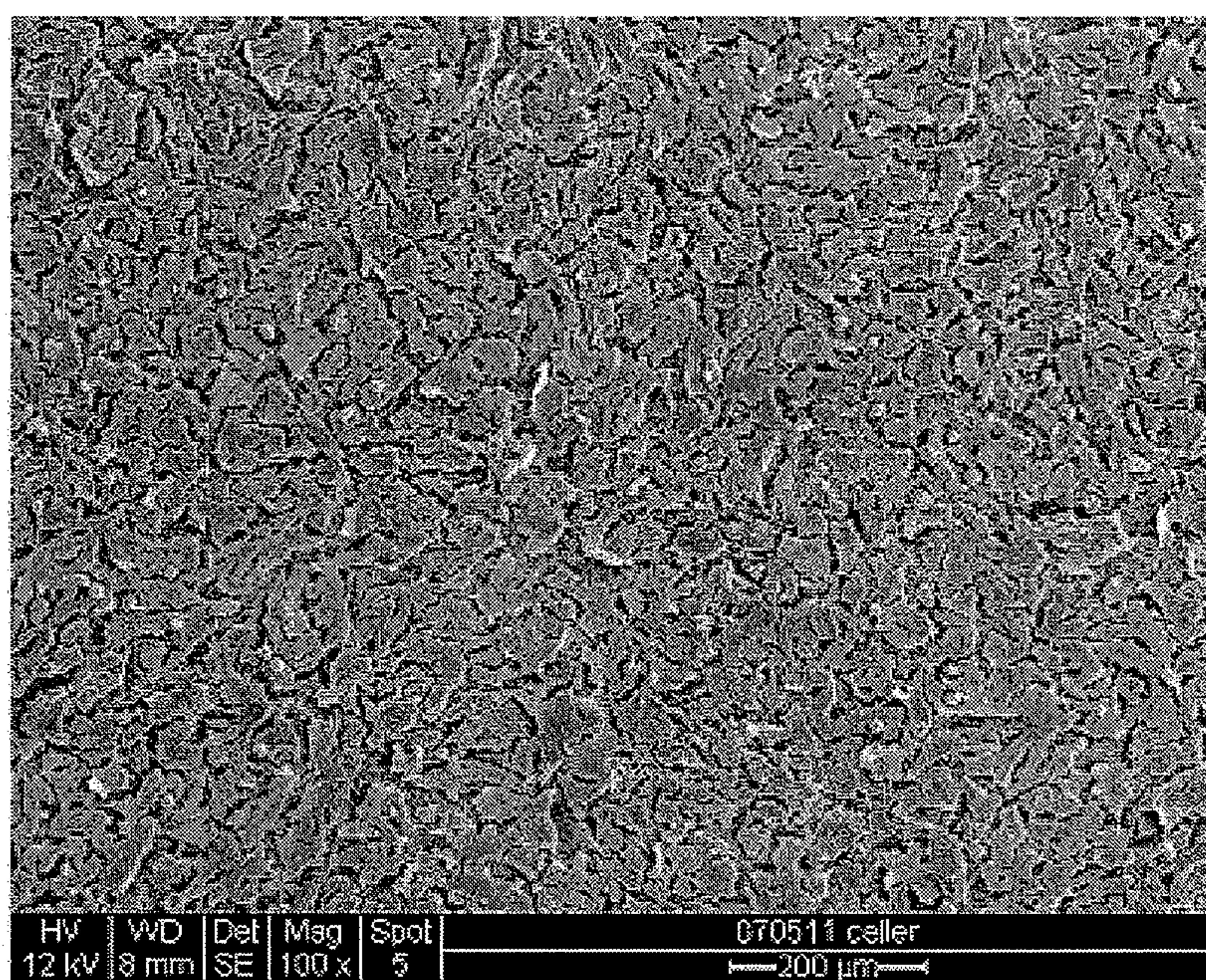
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*Fig. 8*

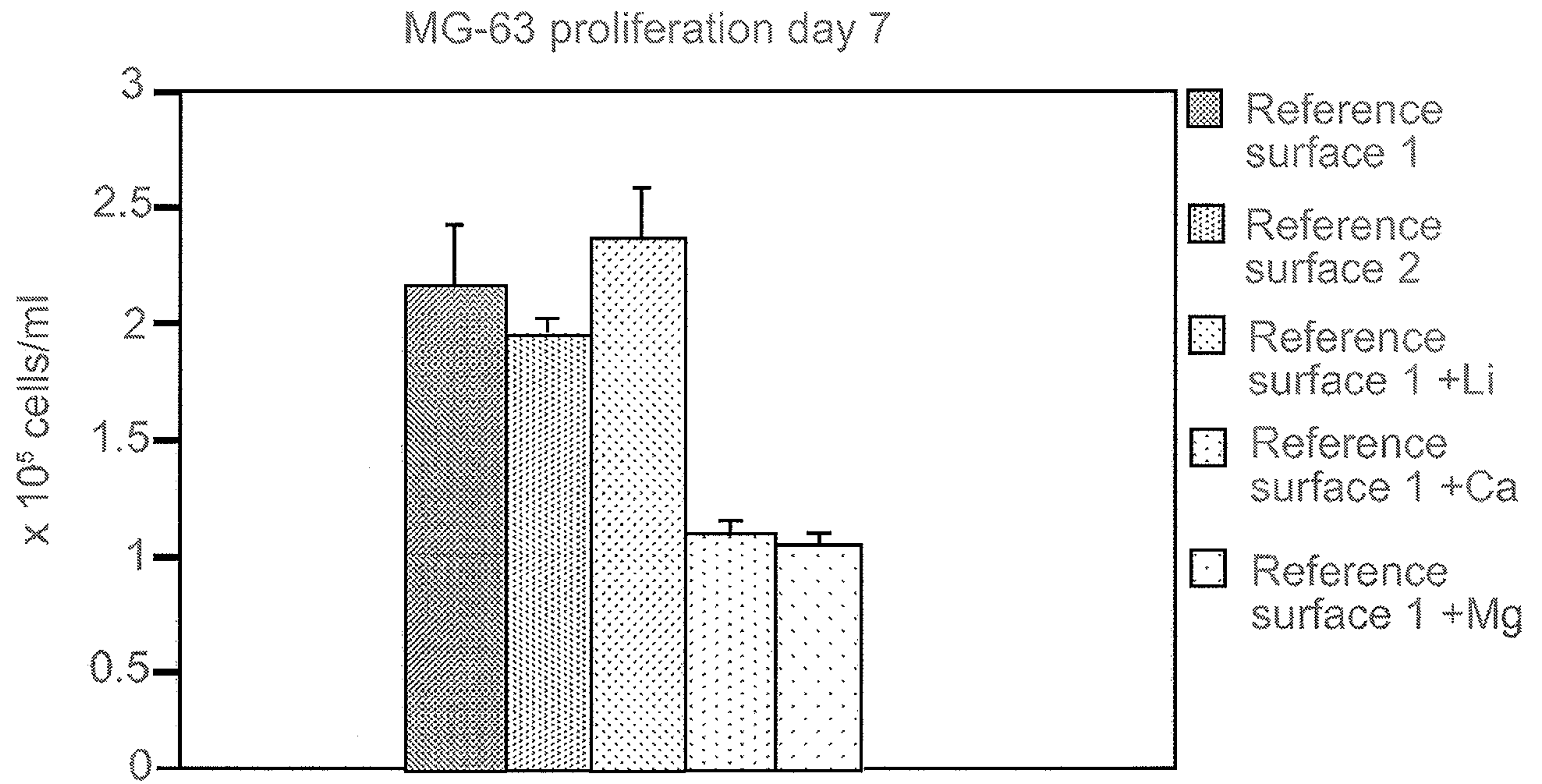
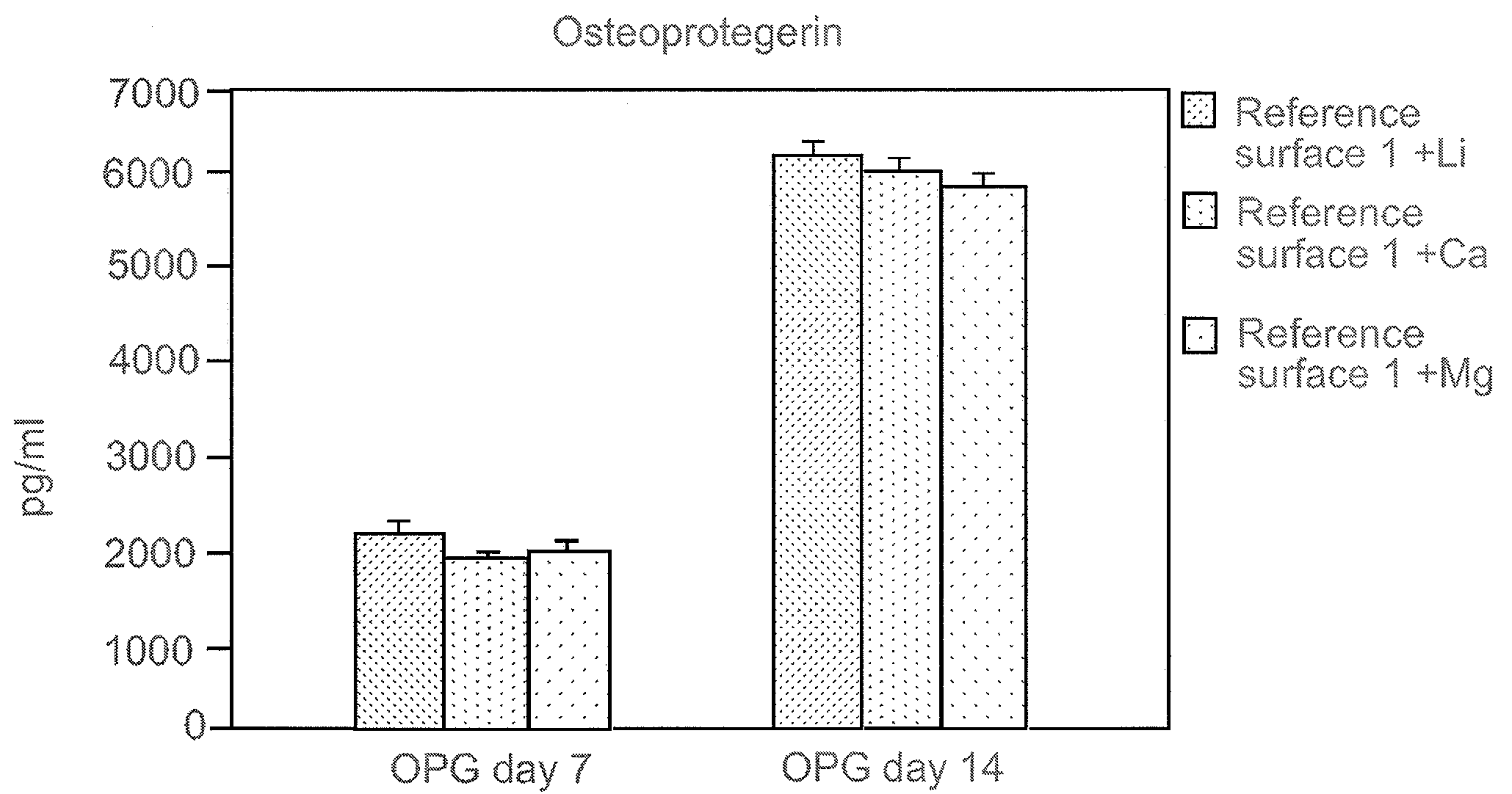
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*Fig. 9*

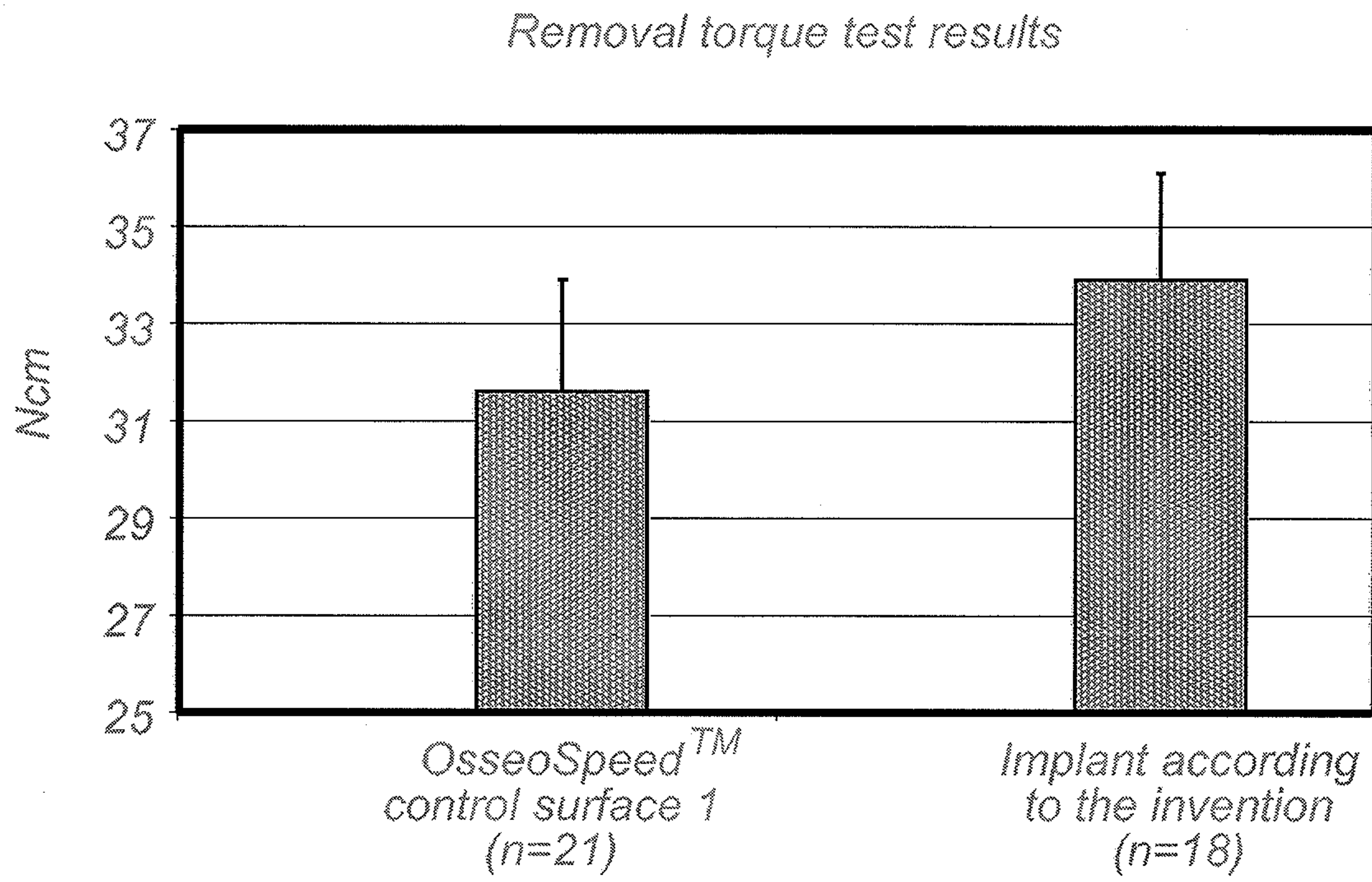
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*Fig. 10*

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*Fig. 11**Fig. 12*

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*Fig. 13*

MG-63 proliferation day 7

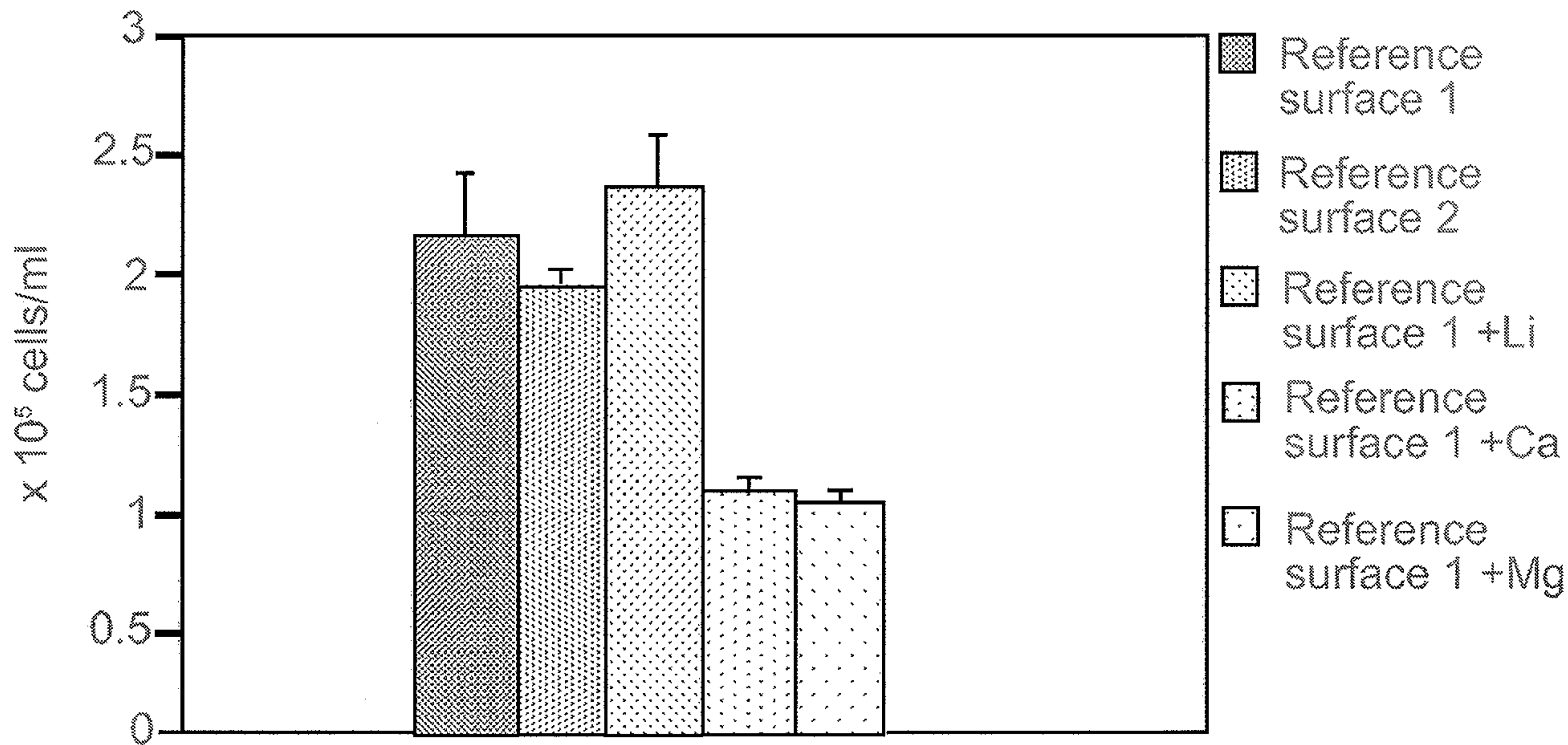


Fig. 11