

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**THE GROWTH OF MAGNESIUM SUBSTITUTED HYDROXYAPATITE ON
(Ti,Mg)N THIN FILMS AND INVESTIGATION OF THEIR POTENTIAL AS
HARD TISSUE IMPLANT MATERIAL**

Ph.D. THESIS

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Molecular Biology-Genetics and Biotechnology Department

Molecular Biology-Genetics and Biotechnology Programme

DECEMBER 2013

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**(Ti,Mg)N İNCE FİLM YÜZEYİNDE MAGNEZYUM KATKILI HİDROKSİAPATİT
BÜYÜTÜLMESİ VE SERT DOKU İMPLANT MALZEMESİ OLARAK
POTANSİYELLERİNİN BELİRLENMESİ**

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To my family,

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Sakip ÖNDER

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ABBREVIATIONS

TiN	: Titanium Nitride
(Ti,Mg)N	: Titanium Magnesium Nitride
HA	: Hydroxyapatite
Mg-HA	: Magnesium Substituted Hydroxyapatite
SBF	: Simulated Body Fluid
MTS	: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Cell Proliferation Assay)
SEM	: Scanning Electron Microscopy
EDS	: Energy Dispersive X-ray Spectroscopy
XRD	: X-Ray Diffractometer
FTIR	: Fourier Transform Infrared Spectroscopy
SS	: Stainless Steel
Co-Cr	: Cobalt-Chromium

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THE GROWTH OF MAGNESIUM SUBSTITUTED HYDROXYAPATITE ON (Ti,Mg)N THIN FILMS AND INVESTIGATION OF THEIR POTENTIAL AS HARD TISSUE IMPLANT MATERIAL

SUMMARY

Titanium and titanium-based alloys are commonly preferred implant materials and used in hard tissue applications to cover the hard tissue damages occurred because of illnesses and accidents. Statistics shows that 90 % of people older than 40 year-old have degenerative illnesses worldwide. It is estimated that 600.000 hip and 1.4 million knee implants will be used just in USA in 2015. These operations are both expensive and painful for patients. Therefore, longevity of implant materials in the body is important for both economical reasons and patients' comfort. Today, lifetime of implant materials is about 10-15 years and replacement or revision of implant materials is mostly necessary afterwards. A new area called revision surgery has also raised in the world for revision of damaged implant materials. An increase in hip and knee revision is expected in 2005-2030 by 137 % and 607 %, respectively. Besides being expensive, success rate of revised implant is lower than first implementation. Therefore, to supply the implant material demand of the market and improve the features of implant materials, studies have been accelerated in this field.

Stainless Steel (316L SS) and Co-Cr alloys are the first metals used in implementation. However, ions that were released from these metals lead to various illnesses. For example, Ni existence causes skin diseases and Co existence makes carcinogenic effect. Furthermore, these materials have higher modulus than bone, which lead to deficiency in bone structure because of insufficient stress transfer. As titanium and its alloys have high biocompatibility, low elastic modulus and ability to connect with the surrounding tissue (osteointegration), they are commonly preferred in biomedical applications. However, low corrosion and wear resistance of titanium based implants lead to limitations in applications. Despite this, as titanium based materials consist of numerous properties needed for an ideal biomaterial, a special interest has been given to develop wear and corrosion resistant titanium based implant materials.

In order to improve the properties of titanium based implant materials, different coating techniques are commonly used. These coatings could be grouped in two categories such as coatings for improving wear and corrosion properties and coatings for improving osteointegration properties of implant materials. Nitrite coatings are commonly preferred to improve the tribological properties of metallic implant materials and Physical Vapor Deposition (PVD) and Chemical Vapor Deposition (CVD) are two main techniques used for this application. Moreover, TiN is a FDA approved coating and commonly used for implant materials and surgery tools. Coatings that are done to improve the osteointegration properties of implant

materials are one of the key factors that influence the success of implant materials in the body. A good biomaterial should be integrated with the surrounding tissue (osteointegration) easily. Ca-P, especially hydroxyapatite (HA), coatings are very useful and applied method to develop the osteointegration properties of biomaterials. Porous structure of HA enables cells to diffuse to the implant surface, so newly deposited tissue starts to grow from the implant material surface. Further, body fluid that carries nutrients for cell can be easily delivered to the cells via porous structure. In literature, there are studies to improve the properties of HA structure with various ion (Sr, Mn, Mg etc.) substitutions. Incubation in simulated body fluid (SBF) is a cheap and easy method to make the doping of different ions into HA structure possible.

In this study, Mg^{2+} doped TiN thin film coatings were deposited on Ti substrates by using PVD technique. The aim was to produce Ti implants with better osteointegration and corrosion properties and to better understand the effect of Mg^{2+} and surrounding cells in the HA deposition process. Mg^{2+} was chosen for substitution in HA structure since it is a cofactor for many enzymes and used in many reactions in metabolism. In addition, in case a corrosion debris or Mg^{2+} release occurred, no toxic effect would be seen in the body and debris could even be used by the surrounding tissue. In the first part of the study, TiN and 6.4 at % Mg^{2+} doped TiN coatings were produced. This percentage was chosen for HA deposition studies, since a previous study of our group showed that when the Mg^{2+} amount was more than 10 at %, corrosion occurred on the surfaces. Further, Mg^{2+} amount affect the Mg-HA structure ($Ca_{10-x}Mg_x(PO_4)_6(OH)_2$) when it was placed in the structure: When x is more than 5 (28 wt %), the structure become totally amorphous. For this first part of the study, thin film coated surfaces were incubated in 1XSBF and 5XSBF solutions for 4 weeks and 3 days, respectively. For 1XSBF, SEM and EDS analysis showed that homogeneous HA coatings were not produced on both coatings, with ((Ti,Mg)N) or without Mg (TiN), but local HA deposition density was higher on Mg presence. XRD analysis provided important results about the behavior of Mg^{2+} that was deposited in thin film coatings. Mg^{2+} doping resulted in a shifting in TiN peaks to lower diffraction angles but these peaks turned back to their original position after for 7 days in 1XSBF. These results showed that Mg^{2+} release occurred from the surfaces and accelerated HA deposition on the surfaces. Phosphate accumulation on the Mg^{2+} surfaces was also higher than Mg-free surfaces. When incubated in 5XSBF, homogeneous HA coatings were obtained on both surfaces within 3 days. Moreover, a decrease in intensity of PO_4^{3-} FTIR peaks because of poor crystallization of HA structure with the Mg^{2+} substitution and an increase in the granule size of HA structures (i.e. 2-3 times) were obtained. These results are in agreement with the literature and accepted as a proof of Mg^{2+} doping into HA structure. In order to understand Mg^{2+} effect more clearly, Mg-free SBF studies were also done. According to the literature, HA deposition could not occur on the surface of implant materials in the absence of the Mg^{2+} . In this study, HA deposits only formed on the Mg^{2+} doped TiN coatings and clearly showed the positive effect of Mg^{2+} . For cell culture studies, Mg-HA deposits were prepared on low and high Mg^{2+} (4.24 at % and 10.42 at %) doped TiN surfaces. Cell proliferation assay (MTS) showed that while cell proliferation was better on low Mg^{2+} doped surfaces, high Mg^{2+} presence resulted in a decrease in cell number.

In the second part, to better mimic the physiological conditions, cell culture medium in the presence and absence of the cells were used for HA deposition studies. Although SBF simulated the chemical composition of body fluid, it does not contain proteins, amino acids etc. that affect the biomaterial behavior in the body. Besides HA deposition studies in cell culture medium, effect of Mg^{2+} amount on the corrosion and cell effect on HA deposition were also studied for 8 weeks period by preparing Mg^{2+} doped TiN coatings with various Mg^{2+} amount (8.1, 11.31, 28.49 at %). Results showed that though it was difficult to observe HA deposits on TiN surfaces after SBF incubation for 4 weeks, HA deposits were started to be observed on TiN coatings after incubation in cell culture medium for 7 days. These results showed that protein and amino acids were also effective on HA deposition and SBF does not simulate body environment very well. HA deposition only occurred on low Mg^{2+} (8.1 at %) doped TiN surfaces after 7 days, and took more time on other surfaces. Corrosion also occurred on high Mg^{2+} (11.31 and 28.49 at %) containing surfaces after 21 days. EDS analysis showed that there was a periodic decrease and increase in Ca/P ratios on surfaces (2-3 weeks). It was thought that pH changes resulted in dissolution in HA structure. After 42 days, homogeneously HA coated structures were obtained on all surfaces. In the presence of cells, both cells and cell culture medium would effect the HA deposition on surfaces. Cell culture studies conducted on TiN and various Mg^{2+} doped TiN surfaces showed that cell proliferation was better on low Mg^{2+} (8.1 at %) containing surfaces compared with Mg free surfaces and cell proliferation decreased with high Mg^{2+} amounts (more than 10 at %). Therefore, 8.1 at % Mg^{2+} was found to be better for both cell proliferation and corrosion resistance. Mineralized collagen FTIR peaks (amid I, amid II amid III and HA) on TiN and 8.1 at % Mg^{2+} doped surfaces were obtained after a week. A similar cycle in Ca/P ratios were also obtained in cell presence.

In this study, it was proved that low Mg^{2+} (less than 10 at %) doped thin film coatings accelerated HA deposition and enabled natural Mg^{2+} doped HA synthesis on the thin film coatings in SBF. Mg^{2+} that was doped in TiN coatings acted as magnesium source and promoted Mg-HA deposition. HA deposition further improved in cell culture medium, probably because of the additional protein, amino acid etc presence. Corrosion and cell culture studies showed that Mg^{2+} amount greater than 10 at % resulted in a decrease both in corrosion resistance and cell proliferation. In cell culture medium, HA deposition on TiN surfaces seemed better compared with the low Mg^{2+} (8.1 at %) containing surfaces. On the other hand, cell proliferation was better on low Mg^{2+} (8.1 at %) doped TiN surfaces compared with the Mg-free TiN surfaces. Therefore, 8.1 at % Mg^{2+} containing (Ti,Mg)N thin film coated Ti surfaces seemed to be more suitable for hard tissue applications because of positive effect of Mg^{2+} on cell proliferation, corrosion resistance and HA deposition.

(Ti,Mg)N İNCE FİLM YÜZEYİNDE MAGNEZYUM KATKILI HİDROKSİAPATİT BÜYÜTÜLMESİ VE SERT DOKU İMPLANT MALZEMESİ OLARAK POTANSİYELLERİNİN BELİRLENMESİ

ÖZET

Kazalar ve hastalıklar sonucu ortaya çıkan sert doku kayıplarının giderilmesi için titanyum ve alaşımları çok kullanılan malzemelerdendir. Yapılan araştırmalara göre, dünyada 40 yaş üzeri nüfusun %90'ında dejeneratif hastalıklar mevcuttur. 2015 yılı itibarıyla, sadece ABD'de 600 bin kalça ve 1.4 milyon diz implantının kullanacağı tahmin edilmektedir. Bu operasyonlar getirdikleri yüksek ekonomik yükün yanında, hasta için ağırlı ve zordur. Bunun için implantın vücutta ömrünün uzun olması, hem ekonomik açıdan, hem de hasta refahı yönünden önemlidir. Günümüzde kullanılan implant malzemelerin ortalama kullanım süreleri 10-15 yıl arasında değişmektedir. Bu süre sonunda implant malzemelerinin değiştirilmesi veya bakımının yapılması gerekmektedir. Bugün dünyada implantlarda kullanım sırasında oluşan hataların onarımı için revizyon cerrahisi ortaya çıkmıştır. 2005-2030 yılları arasında kalça revizyon cerrahisinde % 137, diz revizyon cerrahisinde %607 artış olması beklenmektedir. Özelliklerini kaybeden implant malzemelerinin bakımı veya değiştirilmesi yüksek maliyetli olmasının dışında, başarı oranı ilk yapılan implantasyona göre çok daha düşüktür. Bundan dolayı hem implant pazarındaki ihtiyacı karşılamak hem de implant malzemelerinin özelliklerini geliştirmek üzere araştırma ve geliştirme çalışmaları büyük bir ivme kazanmıştır.

Paslanmaz çelik (316L SS) ve Kobalt-Krom alaşımları (Co-Cr) metalik implant malzemesi olarak kullanılan ilk malzemelerdir. Fakat bu malzemelerden salınan Ni, Cr ve Co gibi iyonlar çeşitli hastalıklara sebep olmaktadır. Örneğin, Ni varlığının deri hastalıklarına sebep olduğu bilinmektedir. Ayrıca vücutta Co varlığı kanserojen etki yaratmaktadır. Bunların yanında 316L SS ve Cr-Co alaşımları kemiğe göre daha büyük bir elastik modüle sahiptir. Bu farklılık kemikte uygunsuz yüzey gerilimine ve bunun sonucunda kemikte erimeye sebep olmaktadır. Titanyum ve alaşımları, biyouyumluluklarının yüksek olması, vücut içerisinde yüksek korozyon direncine sahip olmaları, kemikle hızlı bir şekilde bütünleşerek (osteointegrasyon) kalıcı bir bağlanma sağlayabilmeleri ve düşük bir elastik modüle sahip olmalarından dolayı biyomedikal uygulamalarda tercih edilmektedirler. Fakat düşük aşınma ve korozyon problemleri titanyum tabanlı implant malzemelerinde de devam etmektedir, dolayısıyla kalça, diz protezleri gibi uygulamalarda kullanımları sınırlıdır. Titanyum inert olması ve elastik modülünün kemiğe daha yakın olmasından dolayı implant malzemeleri açısından çok uygundur. Bu sebeple titanyum tabanlı implant malzemelerin aşınma ve korozyon dirençlerini geliştirilmeye yönelik çalışmalar oldukça yaygındır.

Titanyum tabanlı implant malzemelerin özelliklerinin iyileştirilmesi için çeşitli kaplama teknikleri yaygın olarak kullanılmaktadır. Bu kaplamalar aşınma ve korozyon özelliklerini geliştirmek üzere yapılan ve osteointegrasyonu geliştirmek üzere yapılan kaplamalar olarak iki sınıfta toplanabilir. Nitrür kaplamalar Ti metallerinin özelliklerini geliştirmek üzere sıklıkla tercih edilen kaplama çeşididir. Nitrürleme çalışmaları Fiziksel Buhar Biriktirme Yöntemi (PVD) ve Kimyasal Buhar Biriktirme Yöntemi (CVD) ile yapılabilmektedir. TiN kaplamalar FDA onaylı bir kaplama çeşidi olup, implant malzemelerinde ve cerrahi operasyon aletlerinde sıkça tercih edilmektedir. Osteointegrasyonu arttırmak üzere yapılan kaplamalar bir implant malzemesinin başarısını belirleyen en önemli faktörlerin başında gelmektedir. İyi bir implant malzemesinin çevre doku ile hızlı bir şekilde bağlanması gerekmektedir. İmplant yüzeyinin Ca-P bileşikleri ile kaplanması osteointegrasyonu arttırmak için kullanılan en yaygın yöntemlerin başında gelmektedir. Hidroksiapatit (HA) bir Ca-P bileşiği olup, kemiğin yapısında bulunan doğal bir mineraldir. Gözenekli yapısı sayesinde hücrelerin implant malzemesinin yüzeyine difüz etmesine olanak sağlayarak, yeni oluşan dokunun doğrudan malzeme yüzeyine bağlanmasına yardımcı olur. Bunun yanında hücreler için gerekli besin maddeleri de kolayca hücrelere ulaşabilir. Günümüzde HA yapısına çeşitli iyonlar (Sr, Mn, Mg vb.) katılarak HA özelliklerini geliştirilmeye yönelik birçok çalışma mevcuttur. Yapay vücut sıvısı (SBF) ile yapılan çalışmalar ile HA yapısına çeşitli iyonların katılabilmesi gösterilmiştir. Örneğin SBF içerisindeki kimyasalların oranları sabit tutularak, Mg^{2+} miktarı değiştirilmiş ve Mg^{2+} iyonunun Ca ile yer değiştirmesi sağlanmıştır.

Bu çalışmada, Ti altlık malzemeler üzerinde ark PVD yöntemiyle Mg^{2+} katkı TiN ince film kaplamalar üretildi. Böylece hem titanyum esaslı malzemelerin korozyon ve osteointegrasyon özelliklerinin geliştirilmesi, hem de Mg^{2+} un ve fizyolojik ortamda bulunan hücre varlığının HA çökmesi üzerine etkisinin ortaya çıkarılması hedeflendi. Mg^{2+} birçok enzimin kofaktörü olması, metabolizmada yaygın bir şekilde kullanılması ve kemik hücrelerinin büyümesi üzerine pozitif etki yapmasından dolayı tercih edilmiştir. (Ti,Mg)N ince film kaplamaların korozyona uğraması veya Mg^{2+} salımı vücutta herhangi bir toksik etkiye sebep olmayacak, oluşan birikinti vücut tarafından kullanılabilir. Daha önce grubumuz tarafından yapılan ön çalışmada at % 10'dan fazla Mg^{2+} katılanmış TiN yüzeylerinde korozyon gözlemlenmiştir. Bundan dolayı çalışmanın ilk kısmında TiN ve 6.4 at % Mg^{2+} katkı TiN ince film kaplı yüzeyler üretildi. Ayrıca Mg-HA yapısındaki Mg^{2+} miktarı HA kristal yapısını da etkilemektedir. $Ca_{10-x}Mg_x(PO_4)_6(OH)_2$ yapısındaki $x > 5$ (28 wt %) olduğunda HA yapısı tamamen amorf olmaktadır. Dolayısıyla at % 6.4 Mg^{2+} içeriği hem HA yapısına girdiğinde HA yapısının amorf yapıya dönüştürmemesi hem de düşük korozyona sebep olmasından dolayı HA çalışmaları için uygun bir miktar olarak kabul edilmiştir. Mg^{2+} katkı TiN ((Ti,Mg)N) ve TiN yüzeyleri öncelikle 1XSBF içerisinde 4 hafta boyunca inkübe edilmiştir. Numuneler üzerinde haftalık analizler yapılmıştır. SEM ve EDS analizleri bir aylık süre sonunda her iki yüzeyde de homojen bir şekilde HA yapısının üretilmediğini göstermiştir. Fakat Mg^{2+} katkı yüzeylerde TiN yüzeylere göre lokal olarak çok daha fazla miktarda HA çökeltisinin olduğu gözlemlenmiştir. XRD analizleri ince filmler içerisine katılan Mg^{2+} un davranışı hakkında önemli sonuçlar ortaya çıkarmıştır. Mg^{2+} katkı ince film içeren yüzeylere ait XRD analizleri, TiN piklerinin Mg^{2+} katkısından dolayı daha düşük kırınım açılarına kaydığını göstermiştir. Fakat bir haftalık 1XSBF inkübasyonundan

sonra Mg^{2+} katkılı yüzeylere ait TiN pikleri tekrar orijinal konumuna geri dönmüştür. Bu sonuç bize (Ti,Mg)N yüzeylerinin Mg^{2+} kaynağı olarak davrandığını ve HA oluşum sürecini hızlandırdığını göstermiştir. FTIR analizleri Mg^{2+} katkılı TiN yüzeylerinde fosfat birikiminin olduğunu fakat TiN yüzeylerinde fosfat birikiminin olmadığını göstermiştir. İnce film kaplı yüzeylerde çökelmiş olan HA yapısının karakterizasyonunun daha iyi yapılabilmesi ve hücre kültürü çalışmaları için homojen HA kaplı yüzeylere ihtiyaç vardır. 5XSBF çalışmaları ile 3 günde her iki yüzeyde de homojen bir şekilde çökelmiş HA yapıları elde edilmiştir. Yapılan analizlerde literatürde HA yapısına Mg^{2+} katkılanmasının göstergesi olarak kabul edilen sonuçlar elde edilmiştir. HA yapısı içerisine Mg^{2+} katkılanması HA yapılarının granül boyutlarının 2-3 kat artmasına ve FTIR PO_4^{3-} piklerinin yoğunluğunun düşmesine sebep olmuştur. PO_4^{3-} piklerinin yoğunluğunda meydana gelen bu düşmenin sebebi literatürde HA yapı içerisine Mg^{2+} girmesi sonucu HA kristal yapısındaki azalma olarak açıklanmaktadır. Kaplamalar içerisine katkılanan Mg^{2+} etkisini daha iyi incelemek amacıyla Mg^{2+} içermeyen 5XSBF çalışmaları da yapılmış ve Mg^{2+} etkisi açıkça ortaya konulmuştur. Mg^{2+} içermeyen SBF çalışmaları sonucunda (Ti,Mg)N yüzeylerinde HA çökeltisi oluşurken, TiN yüzeylerinde HA çökelmesinin oluşmadığı gözlemlenmiştir. Yapılan SEM, XRD ve FTIR analizleri de Mg^{2+} li yüzeylerde HA varlığını doğrulamış, TiN yüzeylerinde HA varlığına dair herhangi bir veriye rastlanmamıştır. HA yapısı içerisine giren Mg^{2+} un hücre büyümesi üzerine etkisini incelemek üzere kemik hücreleri kullanılarak hücre kültürü çalışmaları da yapılmıştır. Hücre kültürü çalışmalarında kullanılmak üzere düşük (at % 4.24) ve yüksek (at % 10.42) Mg^{2+} içeriğine sahip iki farklı (Ti,Mg)N ince film kaplı numune hazırlanmıştır. Daha sonra hazırlanan numuneler 5XSBF içerisinde inkübe edilmiş ve Mg-HA yapıları ile kaplanmıştır. Kontrol olarak yine TiN yüzeylerinde çökelen HA yapıları kullanılmıştır. MTS çalışmaları hücrelerin at % 4.24 Mg^{2+} katkılı TiN yüzeylerinde oluşan Mg-HA yapıları üzerinde HA yüzeylere göre çok daha iyi büyüdüğünü göstermiştir. Fakat yüksek Mg^{2+} katkılı TiN yüzeylerinde sentezlenen HA yapısı üzerinde hücrelerin büyümesi yavaşlamıştır. Dolayısıyla düşük Mg^{2+} varlığı hücre büyümesi üzerinde pozitif bir etki yaratırken, yüksek Mg^{2+} hücre büyümesini negatif yönde etkilemiştir.

Çalışmanın ikinci kısmında fizyolojik ortamı daha iyi taklit edebilmek için SBF yerine hücre kültürü besiyeri kullanıldı. Çünkü SBF içerik olarak vücut sıvısını tam olarak taklit edememektir. Vücut içerisinde yer alan proteinler, amino asitler, hücreler vb. hepsi implant malzemenin fizyolojik ortamda davranışını, dolayısıyla HA oluşum sürecini etkiler. Bu kısımda HA oluşumunun yanında malzemelerin korozyon özellikleri de çalışıldı. Bunun için farklı Mg^{2+} içeriğine sahip (at % 8.1, 11.31, 28.49) (Ti,Mg)N ve kontrol olarak TiN ince film kaplamalar üretildi. Numuneler hücre varlığında ve yokluğunda 8 haftalık bir periyotta hücre kültürü besiyeri içerisinde inkübe edildi. Sadece hücre kültürü besiyeri ile yapılan çalışmalar daha ilk hafta sonunda SBF çalışmalarına göre farklılıklar ortaya koydu. SBF çalışmalarında TiN yüzeylerinde HA çökmesi ancak 4 hafta sonunda gözlemlenirken, hücre kültürü besiyeri ile yapılan çalışmalarda numune yüzeylerinde HA çökmesi ilk hafta sonunda gözlemlendi. Bu sonuç protein ve amino asitlerin HA çökmesinde ne kadar etkili olduğunu ortaya çıkarmıştır. Mg^{2+} katkılı yüzeylerde ilk hafta sonunda sadece at % 8.1 Mg^{2+} katkılı (Ti,Mg)N yüzeylerinde HA çökmesi gözlemlenirken, diğer yüzeylerde 2. ve 3. haftalarda HA çökeltisi gözlemlenmeye başlamıştır. Ayrıca yüksek Mg^{2+} katkılı TiN yüzeylerinde (11.31 ve 28.49 at %) 21. günden itibaren yüksek korozyon meydana geldi. Yüzeylerde yapılan

EDS analizleri periyodik (2-3 hafta) bir şekilde yüzeylerde oluşan Ca/P oranlarında bir düşme ve yükselme olduğunu gösterdi. Bunun muhtemel sebebi, ortam pH değerinde meydana gelen değişikliğe bağlı olarak kristalize olamayan HA yapılarında meydana gelen çözülmeler olarak düşünülmüştür. TiN ve bütün (Ti,Mg)N ince film kaplı yüzeylerde 42. günden sonra homojen bir şekilde çökelmiş HA yapıları elde edildi. Hücresiz ortamda elde edilen bu sonuçlardan sonra, besi yeri ortamında ve hücre varlığında yapılan deneylere geçildi. TiN ve çeşitli Mg^{2+} katkılı TiN yüzeylerinde yapılan hücre kültürü çalışmaları ile Mg^{2+} un hücre büyümesi üzerinde etkili olduğu gösterilmiştir. Hücreler düşük Mg^{2+} içeren yüzeylerde TiN yüzeylere göre çok daha iyi büyümüşlerdir. Fakat, Mg^{2+} miktarı arttıkça hücre sayısı da azalmaya başlamıştır. Dolayısıyla at % 8.1 Mg^{2+} miktarı hem hücre büyümesi hem de kaplama korozyon direnci açısından en uygun Mg^{2+} miktarıdır. Mg^{2+} varlığının aynı zamanda mineralizasyonu da etkilediği ortaya çıkmıştır. 1 haftalık hücre kültürü çalışmaları sonucunda TiN ve at % 8.1 Mg^{2+} içeren yüzeylerde mineralize olmuş kolajen yapısına ait (amid I, amid II ve amid III ve HA) FTIR pikleri elde edilmiştir. Yüksek Mg^{2+} içeren yüzeylerde bu pikler 2-3 hafta arasında oluşmaya başlamıştır. EDS analizlerinde Ca/P oranlarının yine periyodik olarak artıp azaldığı gözlemlenmiştir.

Bu çalışmada yapılan SBF çalışmaları ile TiN ince filmler içerisine katkılanan düşük Mg^{2+} (<10 at %) miktarının yüzeylerde HA çökmesini hızlandığı ve yüzeylerde doğal Mg-HA sentezlenebileceği gösterilmiştir. SBF koşullarında yapılan çalışmada ince film içerisindeki Mg^{2+} un magnezyum kaynağı olarak görev yaptığı ve ortama salmış olduğu Mg^{2+} iyonları ile Mg-HA oluşumuna katkıda sağladığı gösterilmiştir. Hücre kültürü besiyerinde yer alan protein, amino asit vb. moleküllerin de HA çökmesinde etkili olduğu ve yapay vücut sıvısının bu tür çalışmalar için fizyolojik ortamı çok iyi taklit edemediği gösterilmiştir. Hücre kültürü besiyeri ve hücreler ile yapılan çalışmalar ayrıca kaplama içerisindeki Mg^{2+} varlığının at %10'dan fazla olmasının hem hücreler üzerinde toksik etki yarattığını, hem de korozyona sebep olduğunu göstermiştir. TiN ince film kaplı yüzeylerde hücre kültürü besiyeri varlığında HA oluşumunun daha iyi olduğu görülmüştür. Fakat fizyolojik ortamda HA çökmesi sırasında hücre varlığı da göz önüne alınmalıdır. Nitekim hücreler at % 8.1 Mg^{2+} katkılı TiN yüzeylerinde TiN yüzeylere göre daha iyi büyümektedirler. Dolayısıyla hücrelerin sevdiği, korozyon direncinin iyi olduğu ve HA çökmesi açısından TiN yüzeylerle benzer özellik sergileyen düşük Mg^{2+} katkılı (Ti,Mg)N ince film kaplı titanyum malzemelerin, TiN ince film kaplı Ti yüzeylere kıyasla sert doku implant malzemesi olarak kullanımı daha uygun görülmektedir.

1. INTRODUCTION

1.1 Purpose of Thesis

Surface properties of implant materials used in hard tissue applications are one of the most important key factors that affect the success of implementation. Titanium and titanium alloys have a wide range of application in orthopedic field because of their valuable properties. However, they also have limitations in their usage as associated with their poor wear and corrosion resistance compared with the stainless steel (SS) and Co-Cr alloys, which are also used hard tissue applications (Geetha et al., 2009). Nevertheless, wear debris of titanium is inert and does not lead inflammatory or allergic effect in the body like SS and Co-Cr alloys. Thus, surface engineering studies are the main focus for improving surface properties of titanium based implant materials.

Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA) coatings are commonly preferred to improve the surface properties of implant materials, as it is a well-known biocompatible material which has similar chemical structure to that of bone minerals (Teixeira et al., 2009). HA could improve the integration of the implant to the surrounding tissue (osseointegration) by providing strong bonds implant-bone interface and, thus, accelerating bone growth from the surface. Further improvement could be obtained by altering the HA properties via doping different ions such as Sr^{2+} , Zr^{2+} , Mn^{2+} , Mg^{2+} (Bracci et al., 2009).

In the present study, our aim is to improve the performance of titanium implant materials for hard tissue applications by surface engineering and the studies were mainly focused on establishing a better understanding in below concepts:

- The effect of Mg^{2+} presence and amount on the HA deposition and corrosion behavior of titanium implant surfaces,
- The potential of (Ti,Mg)N thin film coated implant surfaces to induce a natural Mg^{2+} doped HA deposition (Mg-HA),
- Differences of bone cell (osteoblast) attachment and proliferation on bare Mg^{2+} doped TiN and Mg-HA coated surfaces,

- Effect of osteoblast presence on the HA deposition

1.2 General Information About Biomaterials

Biomaterials are synthetic or natural materials that are used in medical devices or in contact with biological systems to treat damaged tissues or organs to restore their functions, or used to replace them.

Biomaterials that are used in medical field can be grouped into four classes depending on their structural and mechanical properties such as metals, polymers, ceramics and composite materials. Metals have high strength, ductility and resistance to wear, so they are generally preferred in hard tissue applications. However, they have some limitations such as low biocompatibility, corrosion, high stiffness compared to the tissues and they can release ions that may cause allergic tissue reactions (Arola et al., 2010). Polymers are generally preferred in soft tissue applications although they could also be integrated to some hard tissue applications like in the case of knee implants. Polymeric materials have unique properties such as flexibility, good biocompatibility, and lightweight. Moreover, they can be easily manufactured into products with the desired shape (Ramakrishna et al., 2001; Ratner & Bryant, 2004) . Ceramics have good biocompatibility, and they are resistant to corrosion and high compression, but brittleness, low mechanical reliability, difficulties in manufacturing, and inelasticity are some of the drawbacks of ceramics. They are generally used in hard tissue applications like metals because of their structural and mechanical properties (Vallet-Regi, 2010). To handle the drawbacks of metals, ceramics and polymers, composites were developed by using different combinations of these materials. Composites have unique properties and are usually stronger than any of the single materials from which they are made. Therefore, they are also commonly used in biomedical applications (Table 1.1) (Nitesh R. Patel, 2012).

Table 1.1 : Classification and properties of biomaterials (Banerjee, 2012).

Materials	Advantages	Disadvantages	Examples
Polymers (nylon, silicon Rubber, polyester, PTFE, etc)	Resilient Easy to Fabricate	Not strong Deforms with time May degrade	Blood vessels, Sutures, ear, nose, Soft tissues
Metals (Ti and its alloys Co-Cr alloys, stainless Steels)	Strong Tough ductile	May corrode, dense, Difficult to make	Joint replacement, Bone plates and Screws, dental root Implant, pacemaker, and suture
Ceramics (Aluminum Oxide, calcium phosphates, including hydroxyapatite carbon)	Very biocompatible Inert strong in compression	Difficult to make Brittle Not resilient	Dental coating Orthopedic implants Femoral head of hip
Composites (Carbon-carbon, wire Or fiber reinforced Bone cement)	Compression strong	Difficult to make	Joint implants Heart valves

Biomaterial demand has shown a rapid growth with the increasing aging population in the world in order to improve comfort and lifetime. Biomaterials could be used in different parts of the human body such as artificial valves in the heart, stents in blood vessels, replacement implants in shoulders, knees, hips, elbows, ears and orodental structures (Figure 1.1) (Ramakrishna et al., 2001; Ratner & Bryant, 2004; Wang et al., 2011). However, the use of implants materials for spinal, hip and knee replacements are prevalent than other implant materials. It has been estimated that 90% of population over the age of 40 suffers from degenerative diseases (Geetha et al., 2009). A survey made with the data collected on total joint replacements surgery showed that the demand for the total hip replacements will rise by 174 % and total knee replacements will rise 673% by the end of 2030 (Kurtz et al., 2007). Joint replacements, especially hip and knee, may result in weakening of the bones, and inflammation in the bone joints.

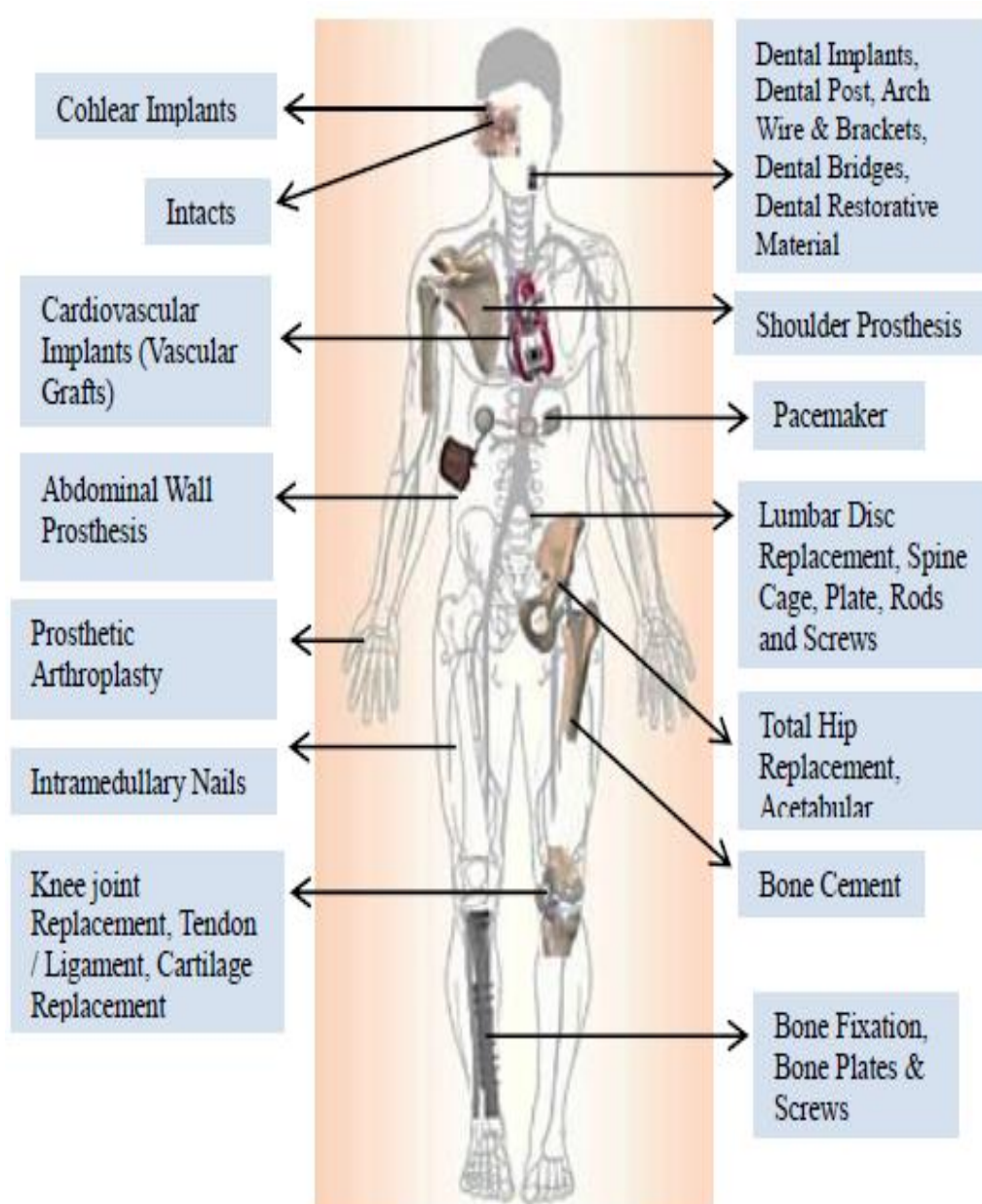


Figure 1.1 : Application locations of different biomaterials (Banerjee, 2012).

Thus, a second surgery was generally needed for the revision of implants. An increase in hip revision surgery by 137% and in knee revision surgery by 607% is expected between 2005 and 2030 (Kurtz et al., 2007). Therefore, studies made to improve the quality of implant materials to prolong its lifetime in body and the demand for new implant materials are expected to increase. Studies to improve the properties of implant materials are very important both for comfort of patient and longevity of implant material. Hard tissue implant materials should be biocompatible, have high corrosion and wear resistance, and low elastic modulus (Ramsden et al., 2007; Singh & Dahotre, 2007). Stainless steel, cobalt chromium

alloys, and titanium-based alloys are the materials used in orthopedic implant applications. However, their high modulus compared with the bone and low wear and corrosion resistance make these materials unsuitable for the long-term usages leading to undesired revision surgery. The main reasons that result in implant material failure are summarized in Figure 1.2.

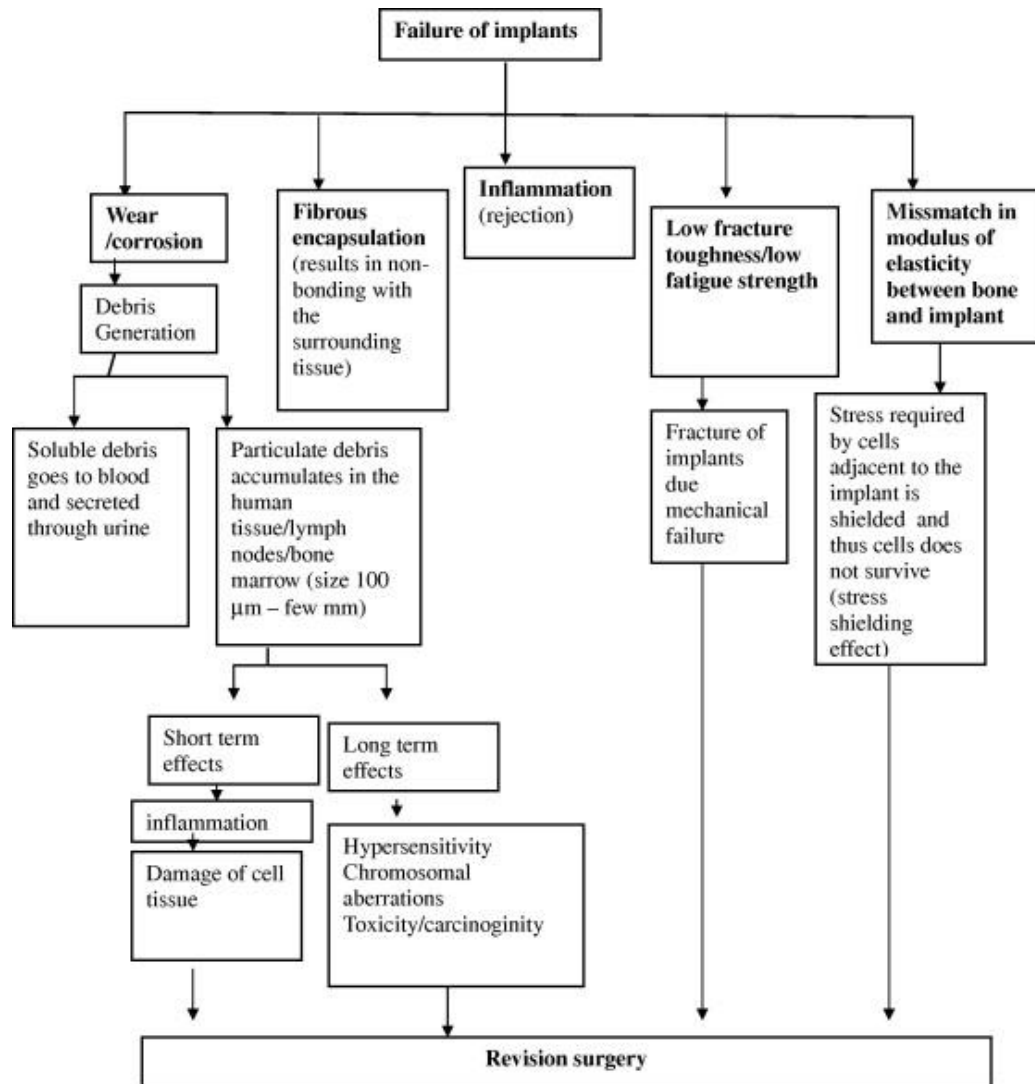


Figure 1.2 : Causes for implant material failure (Geetha et al., 2009).

Current studies mainly focus on the production of implant materials that may serve for longer times without any failure, and do not need any revision surgery. Although several materials are in usage for biomaterial production, titanium based materials are the most-widely preferred materials for many applications because of their valuable properties. However, titanium based materials have also limitations in application and considerable attention has been given in order to handle the drawbacks of these materials.

1.3 Requirements for Metallic Implant Materials

1.3.1 Mechanical properties

Mechanical properties of implant materials change depending on the application type. Properties such as hardness, tensile strength, modulus and elongation are primarily important depending on the type and functionality of a specific implant part (Bauer et al., 2013). If mechanical properties of an implant material do not match that of the bone, the result would be fracture on implant. It is therefore important to choose a biomaterial that has an elastic modulus close to that of bone. Higher stiffness of implant materials results in bone resorption around implant and subsequently loosening. This biomechanical incompatibility results in death of bone cells and called as stress shielding effect (Sumner et al., 1998).

1.3.2 Biocompatibility

The ability of a material to perform with an appropriate host response in a specific application is known as biocompatibility (Williams., 1987). An implanted material must be compatible with human body and should not release toxic ions that may cause any inflammatory or allergic reactions in the human body (Banerjee, 2012). Two important factors that influence the biocompatibility of a material are host response induced by the material and the material degradation. When biomaterial is implanted, it is exposed to body fluids and several reactions take place between the biomaterial and host (Figure 1.3).

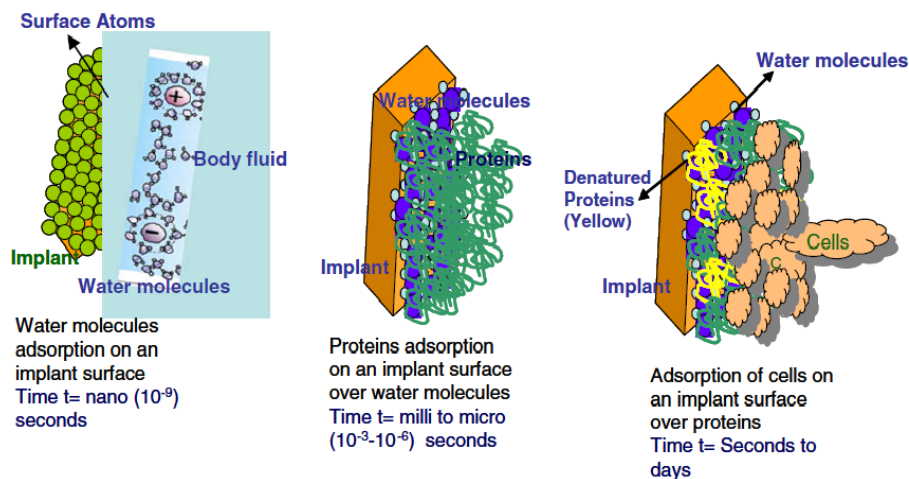


Figure 1.3 : Reactions occurred on implant surfaces after implantation (Geetha, et al., 2009).

The host may give a response to foreign substance after implementation in the localized environment but this response should not result in harmful systematic effects (Geetha et al., 2009).

1.3.3 High corrosion and wear resistance

High corrosion and wear resistance of biomaterials are extremely important in order to decrease ion release rate from biomaterials that cause allergic and toxic reactions on tissues (Hallab et al., 2005). The low wear resistance also results in implant loosening and wear debris. Wear debris is found to cause several reactions in the tissue in which it is deposited (Sargeant & Goswami, 2006). Thus, development of implants with high corrosion and wear resistance is of prime importance for the longevity of the material in the human system.

1.3.4 Osteointegration

Osteointegration was first defined as anchorage of bony tissue around the implant without growth of fibrous tissue at the bone-implant interface (Albrektsson & Johansson, 2001). Any fibrous tissue that is formed at the bone-implant interface results in implant failure, since implant surface cannot integrate with the adjacent bone and other tissues well (Viceconti et al., 2000). Thus, materials with an appropriate surface are highly essential for the implant to integrate well with the adjacent bone without forming a fibrous tissue. Both surface chemistry and surface topography have an important effect on the development of good osteointegration (Ijiri et al., 1996; Nyberg et al., 2013).

1.4 Metallic Implant Materials and Their Limitations

There are three types of materials that are commonly used in surgical implant production such as 316L stainless steel (316LSS), cobalt chromium (Co–Cr) alloys and titanium and its alloys (Geetha et al., 2009). However, all these implant materials have limitations that restricts their usages in medical applications.

1.4.1 Stainless steel (316LSS) and cobalt chromium (Co-Cr) alloys

Stainless steel (SS) and Co-Cr alloys are the first metals used to manufacture metallic implant materials. Stainless steel has high fatigue strength, fracture toughness, easy availability, low cost and good workability (Kato et al., 2013). Though it is mainly preferred in fracture fixation applications, it is also used in the production of

traumatological temporary devices such as fracture plates, screws and hip nails (Navarro et al., 2008). Co-Cr alloys also have excellent corrosion and wear resistance, high elastic modulus, and fatigue strength. Artificial heart valves, dental prosthesis, orthopedic fixation plates, artificial joint components, vascular stents are some applications in medical field where Co-Cr alloys are used (Temenoff & Antonios, 2008). However, both SS and Co-Cr alloys have limitations as metallic implant material that restricts their area of usage.

SS and Co-Cr alloys release toxic ions such as Ni, Cr and Co in physiological conditions because of corrosion (Okazaki & Gotoh, 2005). Studies in literature showed that presence of Ni ions resulted in skin related diseases associated with Ni toxicity (Burlibasa et al., 2009; Geetha et al., 2009). Moreover, numerous animal studies have shown the relation of Co ion presence in carcinogenicity (McGregor et al., 2000). In addition, both 316L SS and Cr-Co alloys have higher modulus than bone. Difference in modulus leads to insufficient stress transfer to bone and cause bone resorption. Therefore, implant materials need revision surgery after some years of implantation.

1.4.2 Titanium and titanium alloys

Titanium and titanium-based materials are the most preferred implant materials because of their numerous desired properties. They have high strength, low density, low corrosion rate, enhanced biocompatibility, low modulus and high capacity to join with bone and other tissues (Rautray et al., 2011; Wang et al., 2011). Moreover, since they are nearly inert to body environment, they are well tolerated (Geetha et al., 2009). Titanium also forms a very stable passive layer of TiO_2 on its surface. This passive surface both provides superior biocompatibility and protects metals from corrosion, which result in a decrease in corrosion rate (Mohan & Anandan, 2013).

Titanium and titanium alloys have a wide range of applications such as, dental implants and parts for orthodontic surgery; joint replacement and parts for hip, knee, shoulder, spine, elbow and wrist; bone fixation materials like nails, screws, nuts and plates; housing device for the pacemakers and surgical instruments in medical area (Geetha et al., 2009). Commercially pure Ti and Ti-6Al-4V (Ti64) are the most common types of titanium materials used implant applications. However, long-term usage of Ti64 based implant materials has some concerns because Al and V release was shown to result in long-term health problems, such as Alzheimer disease,

neuropathy and osteomalacia (Nag et al., 2005). Further, titanium has poor shear strength, so usage of titanium and its alloys in applications such as bone screws, plates, etc are limited (Long & Rack, 1998). Titanium-based alloys have also a high coefficient of friction and this leads to wear debris accumulation around tissues. Wear debris results in inflammatory reaction causing pain and loosening of implants due to osteolysis so should be avoided (Geetha et al., 2009).

1.5 Wear and Corrosion in Titanium and Titanium Alloys

1.5.1 Wear in titanium and its Alloys

Although titanium is preferred in various medical applications, its poor tribological properties limit its usage in many potential applications (Budinski, 1991). Especially, titanium and its alloys have a low resistance to plastic shearing and Ti64/UHMWPE (ultra-high-molecular weight polyethylene) combination is therefore generally used in TJR (total joint replacements) prosthesis (Oh et al., 2004) (Figure 1.4). Wear studies showed that femoral heads made of titanium alloy are subjected to a considerably high wear (73.4 %). The wear was minimum in Co–Cr alloy, and in between for 316L stainless steel (McGee et al., 2000). In addition to that, hip simulation studies showed that when UHMWPE was combined with Ti64 the wear rate was determined to be 35% higher than that measured for Co–Cr–Mo.

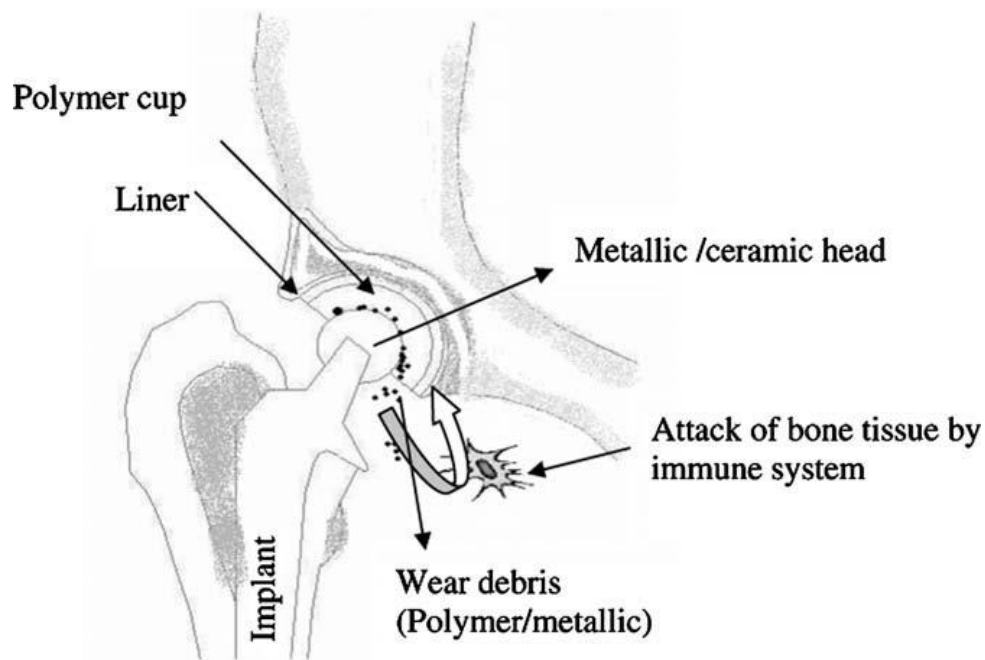


Figure 1.4 : Wear of an implant (Geetha et al., 2009).

Surface oxides are very effective on the wear behavior of the implant materials, so instability of oxide layer because of UHMWPE lead to a decrease in wear resistance. High amount of wear debris in tissues around the prosthesis was also determined to contribute the failure and wear debris accumulation in Co-Cr and SS were lower compared with that of Ti alloys (McGee et al., 2000). On the other hand, one should keep in mind that although wear debris of Co-Cr and SS are in low quantity in the body, their toxicity result in long-term health problems as mentioned before. Thus, there is a great deal of interest to develop wear resistant titanium and titanium alloys.

1.5.2 Corrosion in titanium and its alloys

Corrosion is another important problem of metallic implant materials since the presence of chloride ions and proteins in the body fluid accelerates corrosion. Metallic implant materials could be corroded as a result of different mechanisms such as pitting, crevice and fretting. Pitting is a localized corrosion type and caused by local dissolution of the passive film that result in formation of cavities on metal surfaces (Szklaarska, 1986). Titanium and its alloys are resistant to pitting corrosion but high fluoride solutions used in dental cleaning procedures lead to corrosion damage in titanium based materials (Geetha et al., 2009). Crevice corrosion occurs at shielded sites such as screw/plate interface and under washers. This type of corrosion generally takes place in the presence of chlorides. Fretting corrosion is a general problem of all load bearing orthopedic implant materials. Slight oscillations of two closely packed surfaces result in fretting corrosion.

The corrosion rate of metallic implant materials is substantially reduced via protective oxide layer on their surfaces. Passive film resistance strongly depends on composition of the film structure, so any change in the ionic composition of the film affects corrosion behavior of the passive film. For example, while vanadium oxide dissolves and results in vacancies in the oxide layer in Ti64 alloy, using Nb as alloying element for titanium increases the stability of surface film and increases passivation (Kobayashi et al., 1998). Presence of proteins may either inhibit or accelerate the corrosion rate of implant materials in the body environment. Further, repassivation feature of an implant material is also important for corrosion rate since during the repassivation process different ions could be incorporated into repassivated layer. These incorporated ions may be very effective in defining the corrosion behavior of implants.

1.6 Surface Modification of Titanium and Titanium Alloys for Medical Applications

In order to improve wear and corrosion resistance and osteointegration properties of implant materials, surface coatings are the most preferred method and discussed in detail in the following section.

1.6.1 Coatings for enhanced wear and corrosion resistance

Ion implantation (Fukumoto et al., 1999), plasma spray coating (Liu et al., 2004), thermo chemical surface treatments such as nitriding (Geetha et al., 2009), carburization and boriding are some of the techniques that have been used to improve the surface hardness of titanium alloys.

TiN coating is a FDA approved coating and it was found that TiN coated hip and knee implants have better wear resistance and biocompatibility (Alves et al., 2005). Different techniques such as physical vapor deposition (PVD) and chemical vapor deposition (CVD) are in use for the N deposition on substrates. Further, different plasma techniques such as plasma and ion nitriding can be used for nitriding studies (Cetinarslan et al., 2012; Zheng et al., 2013). Nitrogen enriched films deposited on titanium and its alloys increase the passivation rate of surfaces and lead to a better corrosion resistance (Thair et al., 2003). Diamond-like carbon (DLC) coating is another promising technique used in surface engineering applications. It is possible to obtain surfaces with improved tribological properties by reducing friction and increasing wear (Kim et al., 2005). Further, corrosion resistance and biocompatibility of surfaces could be improved via DLC coatings. In literature, it was shown that cell proliferation on DLC coated surfaces are better compared with bare titanium surfaces, and no inflammatory effect or cytotoxicity were observed (Mohanty et al., 2002). Oxygen diffusion hardening (ODH), thermal oxidation and laser annealing techniques have also been used to improve the surface hardness of titanium alloys. Surface hardness of titanium alloys similar to Co-Cr alloys can be obtained after ODH treatment (Zabler, 2011). Laser annealing is another innovative technique which allows tailoring the $\alpha+\beta$ titanium alloys at nano scale via laser and results in improved tribological property (Geetha et al., 2009).

1.6.2 Coatings for high osteointegration

Osteointegration is defined as the process of bone healing and the formation of new bone around biomaterials and success of the implant materials in orthopedic surgery is based on the integration of implant material with the surrounding bone. When the implant materials are in the body, numerous reactions occur on the surface beginning with the absorption of water molecules and proteins. Then, cells start to adhere to the surface and proliferate on the implant materials and differentiate to synthesize osteointegration precursors if the material is compatible with the body. Incompatible material causes an inflammatory response. Further, a fibrous tissue can be formed on the implant material and formed fibrous tissue may result in failure of osteointegration. As mentioned earlier, all these possibilities are depending on chemistry, topography, roughness and energy of the surfaces. Especially, surface energies of implant materials have numerous effects for the success of implementation (Kilpadi & Lemons, 1994).

Different strategies have been used in order to increase the osteointegration of titanium based materials. Surface roughness is known to influence the cell proliferation and morphology and it could be altered easily by physical treatment techniques such as acid etching and sand blasting. These two commonly used techniques allow to obtain surfaces with grooves and depressions that effect the cell orientation and attachment (Boyan et al., 1995). It was showed that grooved surfaces make a positive effect on the cell attachment and proliferation (Jayaraman et al., 2004). Grain size is also important for biomechanical properties of implant materials. In literature, different methodologies such as development of porous surface, coating of nano ceramic particles, thermal heat treatment, HA and oxide coating have been applied to increase biomechanical compatibility and to reduce the grain size (Geetha et al., 2009). Techniques like surface grit blasting and polishing also enhance the cell growth, but change the oxide thickness of the titanium materials. As oxide layer is closely associated with the biocompatibility of titanium materials, this surface treatment leads to a decrease in biocompatibility. Porosity of implant materials is also important for the bone integration. Bone ingrowth into porous surface interlocks the biomaterial with the surrounding bone tissue, and results in a better biomechanical compatibility and resistance to fatigue loading (Geetha et al., 2009).

Calcium phosphate film coatings are commonly used to ensure chemical bonding with the living bone (W. Suchanek & Yoshimura, 1998). Synthetic hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)(\text{OH})_2$, is a calcium phosphate compound and chemically very similar to bone structure. It is commonly used in surface engineering studies to improve biocompatibility since it promotes bone generation (J. Huang et al., 2010) (Figure 1.5).

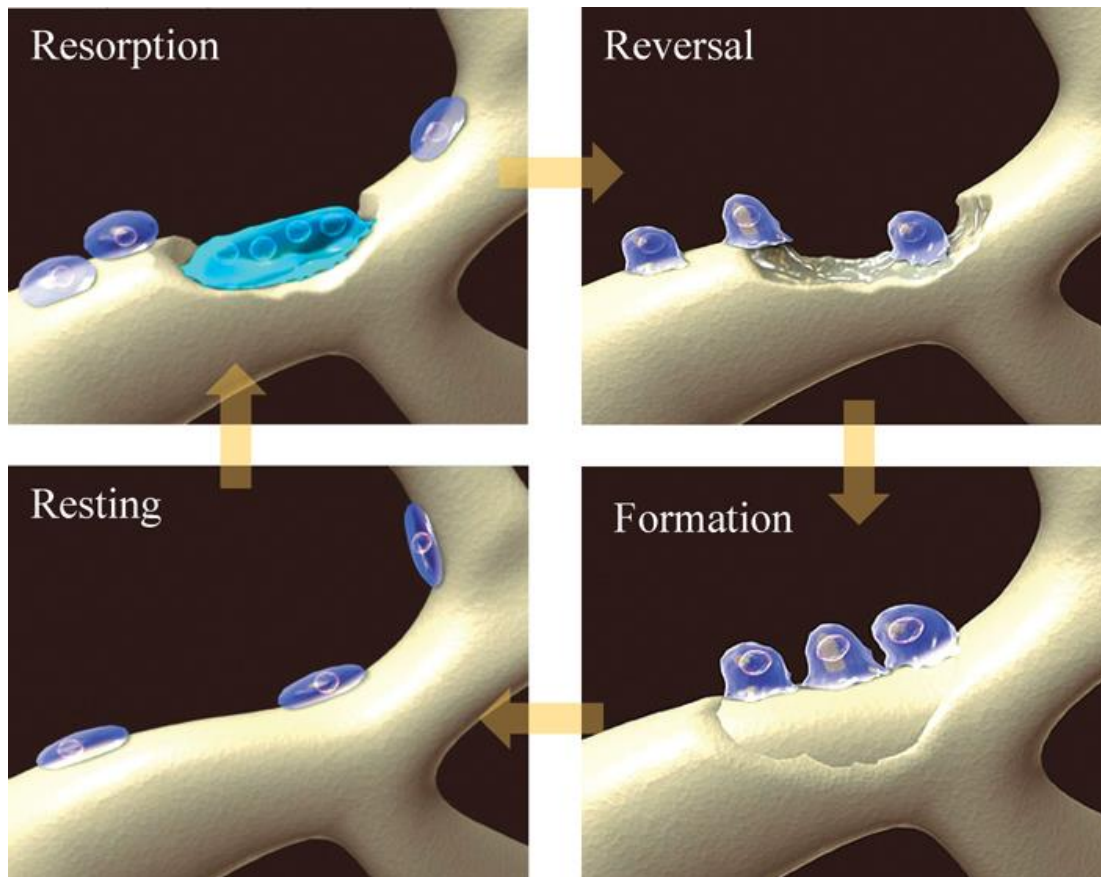


Figure 1.5 : Bone generation process (i) Resorption: stimulated osteoblast precursors release factors that induce osteoclast differentiation and activity. Osteoclasts remove bone mineral and matrix, creating an erosion cavity. (ii) Reversal: mononuclear cells prepare bone surface for new osteoblasts to begin forming bone. (iii) Formation: successive waves of osteoblasts synthesize an organic matrix to replace resorbed bone and fill the cavity with new bone. (iv) Resting: bone surface is covered with flattened lining cells. A prolonged resting period follows with little cellular activity until a new remodelling cycle begins (Coxon et al., 2004).

Preliminary studies conducted with the micro arc oxidation method showed that treatment resulted in porous oxide layer with the incorporated Ca-P ions on the titanium surfaces and lead to a better osteointegration. An increase in alkaline

phosphate (ALP) activity was found with the increased oxide layer thickness and Ca-P ions in the layer (Li L. H., et al., 2004) .

In order to have better osteointegration and cell proliferation, it is also possible to alter the hydroxyapatite structure with different ion substitutions such as Sr^{2+} , Zr^{2+} , Mn^{2+} , Mg^{2+} (Bracci et al., 2009). Different methods such as pulsed laser ablation, sputter deposition, electrophoretic deposition, sol-gel, thermal and plasma spray techniques are used for the Ca-P deposition on the surface of biomaterials (T. Huang et al., 2011; Inagaki et al., 2003; Li et al., 2002; Massaro et al., 2001; Nelea et al., 2004; Roy et al., 2011; van der Wal et al., 2006; Yang et al., 2005; Yang & Ong, 2003). However, structure and chemistry of HA films show a strong dependence on the deposition technique used. Amorphous structure or low crystallinity of deposited HA films remains to be the main issue on the production of HA coatings since it directly affects the biological performance. After the synthesis process, a subsequent heat treatment was conducted to complete the HA formation reactions and crystallization. The heat treatment process conducted in a temperature range of 300–1000 °C may lead to the formation of undesired whitlockite phase (Fadeev et al., 2003).

1.7 Hypothesis

It is considered that improving the surface properties of titanium implant materials for hard tissue applications via (Ti,Mg)N thin film coatings which may enable Mg^{2+} doped HA synthesis in physiological conditions is possible. Simulated body fluids (SBF) are widely used in order to study the properties and kinetics of HA formation on implant surfaces. Detailed reviews on this subject can be found in the literature (Li et al., 2004; Ohtsuki et al., 1992; Suchanek, et al., 2004). Since SBF contain Mg ions, its substitution for Ca ions is expected to some extent during the HA formation process. In this context, Suchanek et al. and Barrere, et al. synthesized Mg- HA using excessive amount of Mg and studied consequent structural and chemical changes on HA. Although this has given valuable insights on the effect of Mg^{2+} ion concentration on HA formation and properties, high magnesium containing solutions fail to simulate the actual body environments so could not be used to comment on HA formation *in vivo* conditions.

Relying on the positive role of Mg^{2+} ions on osteointegration of implants and growth kinetics of HA, two approaches could be used for the increased availability of Mg^{2+} ions on implant surfaces:

1. Using Mg containing alloys as implant materials that have ability to release Mg in a controlled manner into the body fluid. However, pure magnesium and Mg based alloys are not suitable materials for implants since they have low corrosion resistance in most electrolyte mediums (Li L. C. et al., 2004; Staiger et al., 2006) limiting their usage in the human body environment.
2. Deposition of bio-compatible coatings containing a desired amount of magnesium on implant materials to give the implant surface a “magnesium releasing ability” in a controlled manner.

The methodology and hypothesis of this study is based on the second approach; namely modifying the implant surfaces with magnesium containing coating that will contribute to HA formation on the implant surfaces in the body environment. For this purpose, a biocompatible coating that can be alloyed with magnesium is required.

As the biocompatible coating, TiN, which is a widely used coating in biomedical applications, was selected. In order to dope TiN with magnesium, cathodic arc physical vapor deposition technique was utilized.

Moreover, in order to better mimic the physiological conditions and predict the HA formation behavior *in vivo* condition, HA deposition studies were also conducted in cell culture medium in the presence and absence of the cells. By that, a preliminary understanding on the effect of cells, amino acids, proteins etc, on HA deposition was obtained. This comparative study was designed to obtain valuable results on the HA deposition behavior in different medium conditions and was the first study to show the effect of cell presence on HA formation on different Ti implant surfaces.

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2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Titanium plate, cathodes and chemicals

Titanium plate, cathodes and chemicals that were used in this study are given in Appendix A along with their suppliers.

2.1.2 Solutions and buffers

The compositions and preparation of buffers and solutions are given in Appendix B.

2.1.3 Laboratory equipment

The laboratory equipment used during the thesis is listed in Appendix C.

2.2 Methods

This study contains two parts. In the first part, Magnesium substituted hydroxyapatite (Mg-HA) was synthesized in different simulated body fluid (SBF) concentrations and characterization of samples after incubation was conducted (Onder et al., 2013). In the second part, in order to better mimic the physiological environment, cell culture medium was used in HA deposition studies instead of SBF. Since *in vivo* conditions also contain cells during the HA deposition process, HA deposition studies in the presence and absence of cells were also done. These studies also revealed information about effect of Mg^{2+} on the cell proliferation and corrosion resistance of thin film coatings.

2.2.1 Simulated body fluid studies (SBF)

2.2.1.1 Deposition of TiN and (Ti,Mg)N coatings

Firstly, titanium samples (15x15x1 mm) for coating process were prepared by using grade 2 Ti plate. Then substrates were polished using 1000, 2500 and 4000-grit SiC papers sequentially. Shortly after the polishing, the substrates were cleaned in acetone and ethanol in an ultrasonic bath for 10 minutes. TiN and Mg^{2+} doped TiN coatings were deposited on samples by using cathodic arc physical vapor deposition

technique. Previous results obtained in our group (Demirel, 2003) on the corrosion resistance of $(\text{Ti}_{1-x}\text{Mg}_x)\text{N}$ revealed that coatings with more than $x = 0.12$ Mg were not stable in chloride containing environments and better corrosion resistance has been achieved with $x = 0.057$. The parameters of the deposition process were, thus, selected to produce coatings containing $x \approx 0.06$ Mg. After cleaning, the substrates were placed in the vacuum chamber then the chamber was evacuated down to 5×10^{-3} Pa. Since evaporation rate of Mg is higher than that of Ti, two Ti and one Mg cathodes were used to adjust Mg content of the coatings. Parameters for $(\text{Ti,Mg})\text{N}$ coatings were given in Table 2.1.

Table 2.1: Parameters used during $(\text{Ti,Mg})\text{N}$ thin film coating.

Cathode	Current (A)	Time (h)	N ₂ (sccm)	Pressure (Pa)	Bias (V)
Titanium	75	1	130	1	-250
Titanium	75				
Magnesium	40				

Before the coating process, the substrate surfaces were heated and etched by Ti ions produced as a result of cathodic arc evaporation. In the ion heating/etching process, a series of high bias voltages were applied on the substrates (-600 , -800 and -1000 V in 30 sec for each step). In order to produce homogeneous coatings, the samples were rotated so that they could be exposed to each cathode for equal time intervals. The schematic of the coating system was given in Figure 2.1.

TiN thin film coatings were also produced as control substrates for cell culture and HA deposition studies. Same procedure without any magnesium cathode was used in this case. In order to set the pressure to 1 Pa, N₂ gas amount were also changed since a decrease in cathode number lead to a change in pressure. Further, coating time for TiN coatings were set to 1.5 h. As atomic radius of Ti is smaller than Mg^{+2} , coating thickness could be thinner in the absence of Mg cathode.

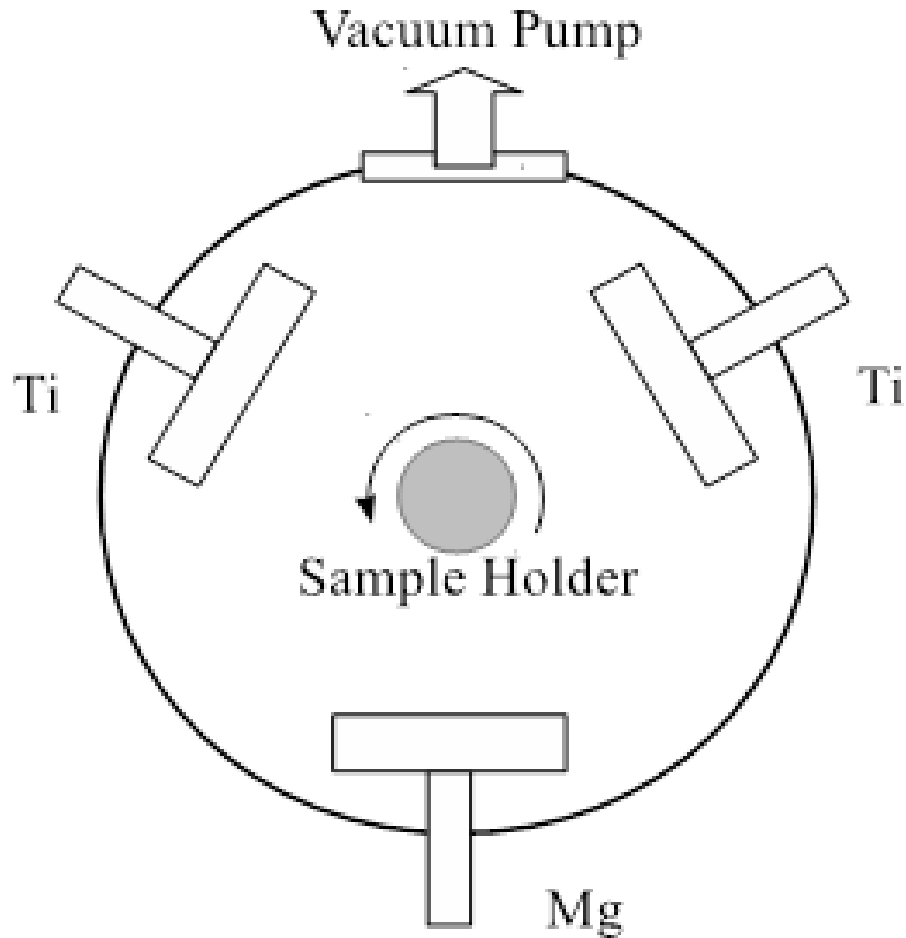


Figure 2.1 : Schematic of the coating system (Onder et al., 2013).

2.2.1.2 Characterization of TiN and (Ti,Mg)N coatings

In order to analyze the structure of thin film coatings different characterization techniques were used. Substrates were kept in a desiccator until the characterization. Thickness of the thin film coatings were measured by using optical profilometer (Appendix C) The magnesium content of the (Ti,Mg)N coatings was analyzed by using scanning electron microscope (SEM) with energy dispersive X-ray spectrometer (EDS) (Appendix C). The phase structure of the coatings and the HA deposits were analyzed by using glancing angle X-ray diffractometer with thin film attachment (Appendix C). The Cu-K α radiation and “ θ scan method” with fixed incidence angle of 0.5° were used.

2.2.1.3 Formation of hydroxyapatite in simulated body fluid

Two different simulated body fluid concentrations (1X SBF and 5X SBF) were prepared as described in the references (Kokubo, 1998) and (Chou et al., 2004).

Chemicals given in Table 2.2 were added into deionized water in the given order. The final pH values of 1X SBF and 5X SBF were adjusted to 7.4 and 6.5, respectively at 37 °C by using 1 M hydrochloric acid or 1 M sodium hydroxide. The samples were incubated in 1X SBF for 1, 2, 3 and 4 weeks in order to monitor the HA formation. In experiments conducted in 5X SBF, HA film formation was accelerated so the samples were kept in the solution for only 3 days. TiN and (Ti,Mg)N coated samples were also tested for 3 days in magnesium free 5X SBF solution to investigate the role of Mg^{+2} on the kinetics of HA formation.

Table 2.2: Chemical composition of 1X and 5X simulated body fluids (Onder et al., 2013).

1X SBF			5X SBF	
Order	Reagent	Amount (gr) in 40 ml solution	Reagent	Amount (gr) in 40 ml solution
#1	NaCl	0.319	CaCl ₂	0.056
#2	NaHCO ₃	0.013	MgCl ₂ .6H ₂ O	0.06
#3	KCl	0.009	NaHCO ₃	0.07
#4	K ₂ HPO ₄ .3H ₂ O	0.009	K ₂ HPO ₄ .3H ₂ O	0.045
#5	MgCl ₂ .6H ₂ O	0.01	Na ₂ SO ₄	0.015
#6	HCl (1M)	1.48 ml	KCl	0.045
#7	CaCl ₂	0.01	NaCl	1.6
#8	Na ₂ SO ₄	0.003	HCl (1M)	Used for adjusting pH to 6
#9	(CH ₂ OH) ₃ CNH ₂	0.24		

2.2.1.4 Characterization of HA deposits

Characterization studies after SBF experiments were also done in order to determine the HA structure. HA deposits formed on the coatings were investigated by using SEM, EDS, XRD, and FTIR spectrometer (Appendix C).

2.2.1.5 Cell proliferation studies on HA and Mg-HA deposits prepared in simulated body fluid (SBF)

In order to determine the effect of Mg^{2+} doping into HA structure, cell proliferation studies were done on previously HA and Mg-HA coated samples. HA and Mg-HA were deposited on TiN and (Ti,Mg)N thin film coatings in 5XSBF after 3 days incubation time (Onder et al., 2013). TiN, 4.24 at % and 10.42 at % Mg^{2+} doped TiN thin film coated surfaces were used for HA and Mg-HA deposition studies. HA and Mg-HA coated titanium samples were firstly sterilized in 70 % ethanol solution (1

h). Human fetal osteoblastic (osteoblast) cells (1000 cells/sample, cell line) were seeded on top of the samples and incubation was done in CO₂ level of 5% and 37 °C. The medium was changed with the fresh one for every 3 days and proliferation of cells on different surfaces were followed by SEM and fluorescence microscopy for 1-7 days.

2.2.1.6 Cell proliferation assay (MTS)

MTS assay was performed on days 1, 4 and 7 days of incubation in order to analyze cell proliferation. All the steps of this experiment were carried out in the dark and all the tubes and plates were covered with aluminum foil. First, the samples were washed with PBS (1X, pH 7.4) and transferred to clean 12-well plates using forceps. Next, in a falcon tube, a solution was prepared with low glucose DMEM (without phenol red) and MTS, having the ratio of 5:1. This solution was added onto the samples (0.75 mL per sample) in the clean 12-well plates and incubated for 2.5 hours in the CO₂ incubator. At the end of incubation, 200 uL of each solution on the samples were transferred to 96-well plate and optical densities were measured at 490 nm using Elisa Plate Reader.

2.2.1.7 Construction of the calibration curve

The media on the cells were removed and the culture flask was washed with PBS (1X, pH 7.4) 2 times. Cells were detached from the culture dish using trypsin (0.25%, 1X) and collected inside a falcon tube. The tube was centrifuged at 1800 rpm for 5-7 minutes. The supernatant was removed and the cell pellet was dissolved with the cell culture media. 10 uL of this cell suspension was put on the hemocytometer and counted. Cells were seeded onto the 12-well plates starting from 10,000 cells to 110,000 cells on separate wells. Low glucose DMEM (without phenol red) and MTS (96[®] AQueous Non-Radioactive Cell Proliferation Assay, Promega, USA) was mixed inside a falcon tube in a ratio of 5:1. This solution was added onto the cells in each well and incubated for 2.5 hours in the CO₂ incubator (Thermo Scientific, Steri-Cycle CO₂ Incubator, USA), in the dark. After that, 200 uL of each solution on the samples were transferred to 96-well plate and optical densities were measured at 490 nm (OD490) using Elisa Plate Reader (Bio-Tek, Elx800, USA). Lastly, an “absorbance vs. cell number” graph was plotted via Microsoft Office Excel.

2.2.1.8 Cell staining (Phalloidin/DAPI)

The samples were double immunostained with Alexa Fluor® 546 Phalloidin and DAPI, which stains the cytoskeleton and nucleus of the cell, respectively for 3 and 7 days of incubation. Firstly, the cell culture media on the samples were removed and the samples were washed with PBS (1X, pH 7.4). For cell fixation, they were kept in 3.7% formaldehyde solution for 30 minutes at room temperature. Washing with PBS (1X, pH 7.4), the samples were incubated inside the staining solution (0.5 mL per sample, Phalloidin in PBS, 1:100 ratio) for 1 hour at room temperature (All the steps after this was carried out in the dark). After washing out the first staining solution, second solution (0.5 mL per sample, DAPI in PBS, 1:1000 ratio) was added onto the samples and kept for 20 minutes at room temperature. Lastly, the samples were washed with PBS (1X, pH 7.4) 3 times and mounted using ProLong® Gold Antifade Reagent (For mounting, one drop of ProLong was added on a microscope slide and the samples were placed on this drop as upside down. The edges of the samples were sealed with nail polish).

2.2.1.9 Characterization of cells

In order to characterize the cells after proliferation, SEM and fluorescence microscopy analysis were carried out. Cells were examined under fluorescence microscope after staining (excitation/emission wavelengths; Phalloidin: 556/570 nm, DAPI: 358/461 nm). For SEM analysis, cells were fixed with cacodylate buffer for 1 hour as described in Appendix B.

2.2.1.10 Statistical analysis

Statistical analyses of the data were performed by Student's t-test in Microsoft Office Excel. Significant difference was considered at the level of $p \leq 0.05$ ($n=3$).

2.2.2 Effect of different parameters on HA deposition, corrosion and cell proliferation

2.2.2.1 (Ti,Mg)N thin film coatings with different Mg^{2+} amounts

TiN and Mg^{2+} doped (Ti,Mg)N coatings were prepared as in 2.2.1.1. However, in order to determine the effect of Mg^{2+} on the corrosion rate and cell proliferation, (Ti,Mg)N coatings with various Mg^{2+} amounts were also prepared in this part. Different arc currents that were used to set Mg^{+2} amounts in (Ti,Mg)N coatings were

given in Table 2.3. TiN thin film coatings were also coated on titanium substrates for control studies.

Table 2.3: Currents used to set Mg^{2+} amount in (Ti,Mg)N thin film coatings.

Ti (1) Cathode (A)	Mg (2) Cathode (A)	Ti (3) Cathode (A)
75	0	70
80-85	15-20	85
80	25-30	80
75	45-50	70

2.2.2.2 Cell proliferation on bare TiN and (Ti,Mg)N thin film coated surfaces

Cell culture studies on bare TiN and (Ti,Mg)N thin film coated surfaces were also done with human osteoblast (hFOB) in order to understand the Mg^{2+} effect on the cell proliferation. The same procedure in section 2.2.1.5 was used for cell culture studies.

2.2.2.3 Statistical analysis

Statistical analyses of the data were performed by Student's t-test in Microsoft Office Excel. Significant difference was considered at the level of $p \leq 0.05$ ($n=3$).

2.2.2.4 Hydroxyapatite deposition and corrosion on substrates

To better mimic the physiological environment of the implant in the body, HA deposition studies were carried out in the presence of osteoblast cells. For this, HA deposition with or without cells were investigated in the absence and presence of Mg^{2+} ions in the TiN and (Ti,Mg)N thin film coatings for 8 weeks period. TiN and different amount of Mg^{2+} doped (Ti,Mg)N coatings were incubated with the cells as

described in section 2.2.1.5. The corrosion of HA was also evaluated but only in the absence of cells.

2.2.2.5 Characterization of TiN and (Ti,Mg)N surfaces after HA deposition and corrosion

SEM and fluorescence microscopy analysis of the surfaces were done in order to observe how Mg^{2+} affect osteoblast cell spreading and morphology. Cell staining protocols explained in section 2.2.1.8 was used for visualization studies. Moreover, HA deposits on both cell free and cell seeded surfaces and corrosion rates of thin film coatings were examined with SEM. EDS analysis of the surfaces were also done to determine Ca/P ratios in the presence and absence of osteoblast cells on the thin film coatings. Finally, FTIR analysis were done to determine HA deposition on samples over a period of 56 days.

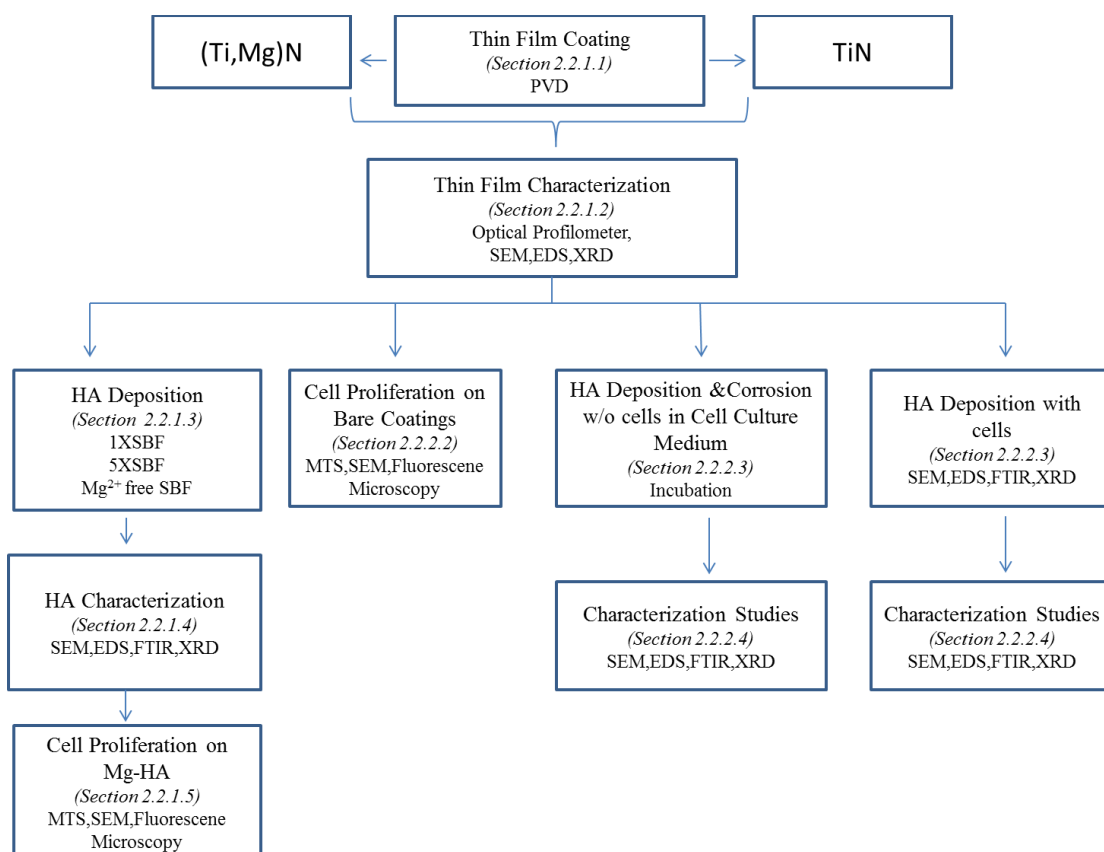


Figure 2.2 : Schematic of studies conducted during thesis.

3. RESULTS AND DISCUSSION

3.1 Characterization of (Ti,Mg)N and TiN coatings

3.1.1 Optical profilometer analysis

The thickness of the coatings that were determined by using optical profilometer were $1.04 \pm 0.1 \mu\text{m}$ and $1.54 \pm 0.3 \mu\text{m}$ for TiN and (Ti,Mg)N, respectively. Measurement points on samples were given in Figure 3.1c. Height difference in the z-direction between Ti ground and thin film coating was taken as thickness of the coatings (Figure 3.1a, b).

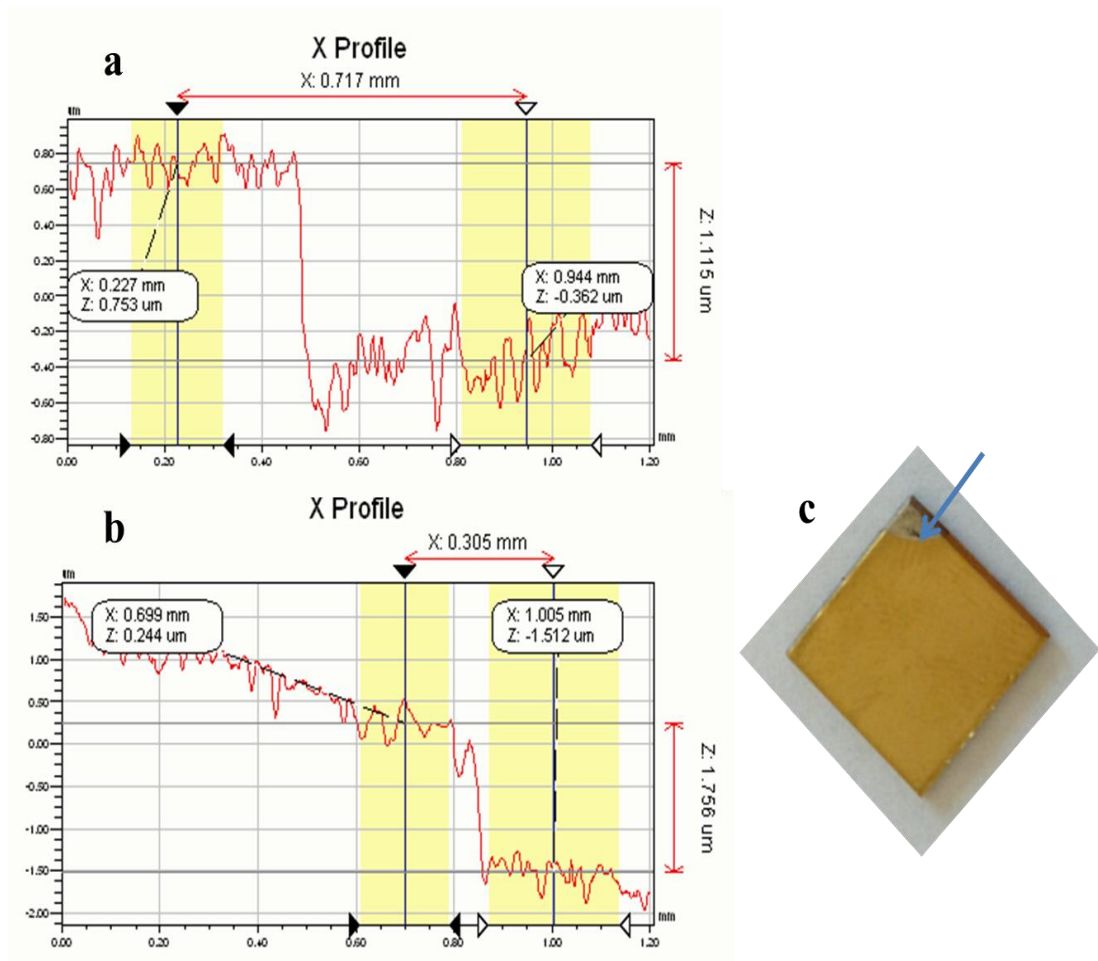


Figure 3.1 : Thickness of thin film coatings (a) TiN, (b) (Ti,Mg)N (c) measurement point.

3.1.2 EDS analysis

In order to determine the Mg^{2+} amount in the thin film coatings EDS analysis were done. Results showed that Mg^{2+} doped TiN thin film coatings contain 6.4 at % Mg and 93.60 at % Ti (Figure 3.2). In a previous study conducted in our group (Demirel, 2003), it was showed that Mg^{2+} amount in thin film coatings should be under 10 at% in order to have high corrosion resistance since low corrosion resistance was observed in physiological conditions above this value. Therefore, 6.4 at % Mg^{2+} amount was chosen for Mg-HA deposition studies in SBF.

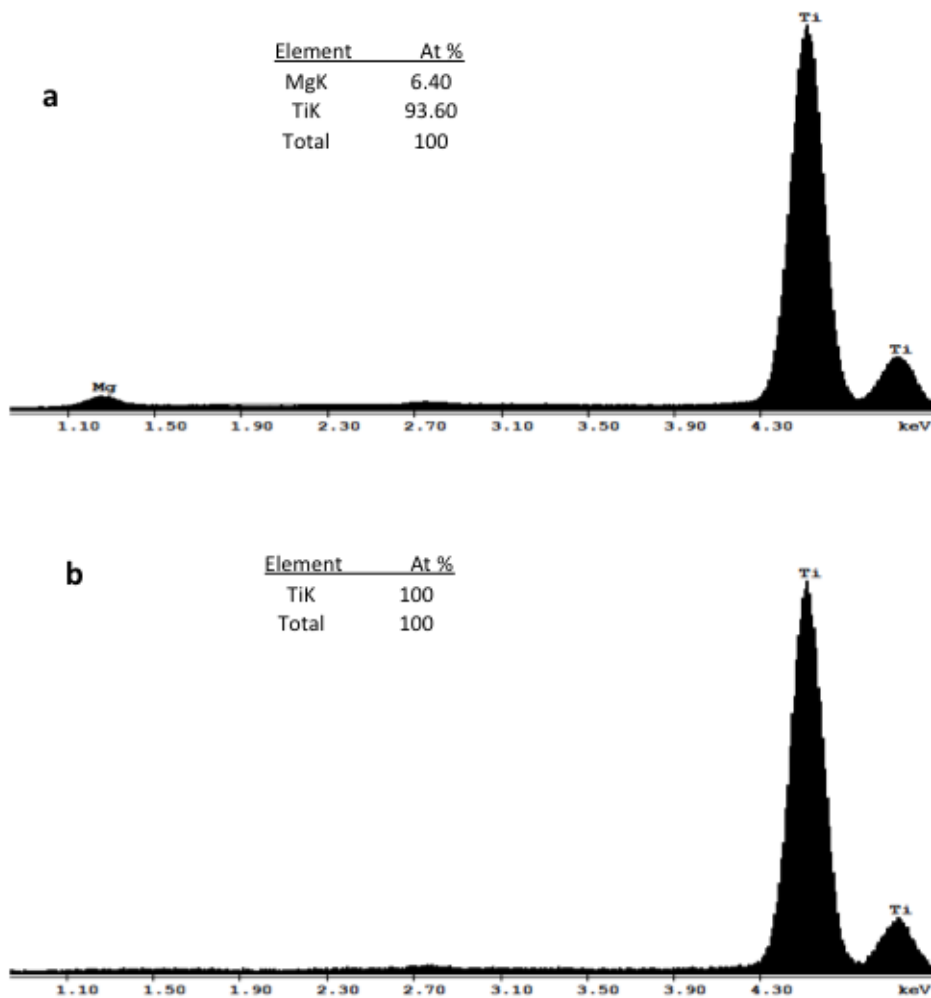


Figure 3.2 : EDS analysis of coatings (a) (Ti,Mg)N (b) TiN.

3.1.3 XRD analysis

X-ray diffraction patterns of TiN and 6.4 at % Mg^{2+} doped TiN coatings were given in Figure 3.3. On both coatings, characteristic TiN peaks at 36.37° (111), 42.43° (200), 61.63° (220) and 73.85° (311) were observed (Figure 3.3a).

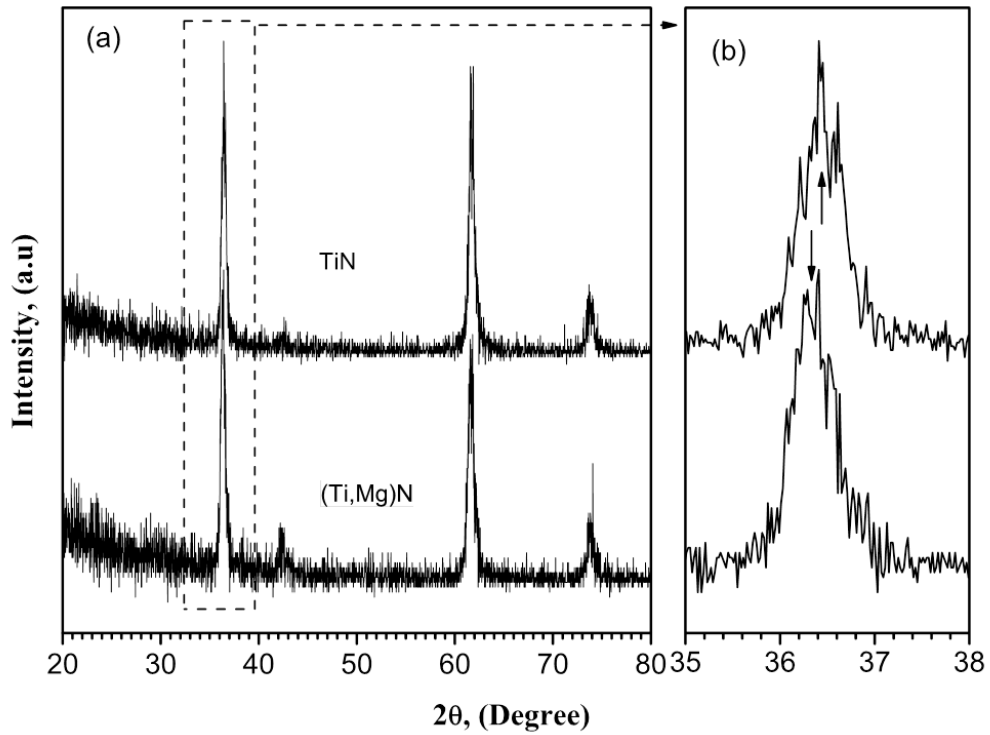


Figure 3.3 : (a) Full range XRD patterns of TiN and (Ti,Mg)N coatings, (b) detailed view of XRD patterns around diffraction peak of (111) crystal plane (Onder et al., 2013).

Furthermore, any diffraction peaks belong to the different phases such as magnesium or magnesium nitride were not present in the XRD pattern of the (Ti,Mg)N sample when the Mg^{2+} amount was around 6.4 at %. These phases and diffraction patterns were observed with the increased Mg^{2+} amount in the previous study (Demirel, 2003). Lattice parameters of the TiN and (Ti,Mg)N coatings were also calculated by using “Philips X’PERT Plus Crystallography and Rietveld Analysis Software”. The calculated lattice parameters of the TiN and (Ti,Mg)N coatings were $a = 4.248 \text{ \AA}$ and $a = 4.253 \text{ \AA}$, respectively. Moreover, it was observed that Mg^{2+} doping into TiN coatings lead to a shift in TiN peaks to lower diffraction angles (Figure 3.3b) expanding the TiN lattice by 0.17 %. Fenker, et al. and Hodroj, et al. reported similar results showing that magnesium substitution for titanium atom in TiN structure resulted in lattice expansion of TiN phase because of higher atomic radius of magnesium.

3.2 Characterization of HA Deposits obtained in 1X SBF

3.2.1 SEM analysis

SEM analysis showed that HA deposits were not homogeneously distributed on the surfaces of both (Ti,Mg)N and TiN thin film coatings after 1X SBF incubation, even after four weeks. Local HA deposits were started to be observed on (Ti,Mg)N surfaces after the first week. HA deposits on the TiN coatings, however, reached to an observable size only after 3rd week (Figure D.1) on certain regions of surfaces. Higher amount of HA deposits could clearly be observed on the (Ti,Mg)N surface at the end of 4th week in the SEM micrograph (Figure 3.4). This result proved the positive role of Mg^{2+} presence on the coating in the initiation of HA deposition.

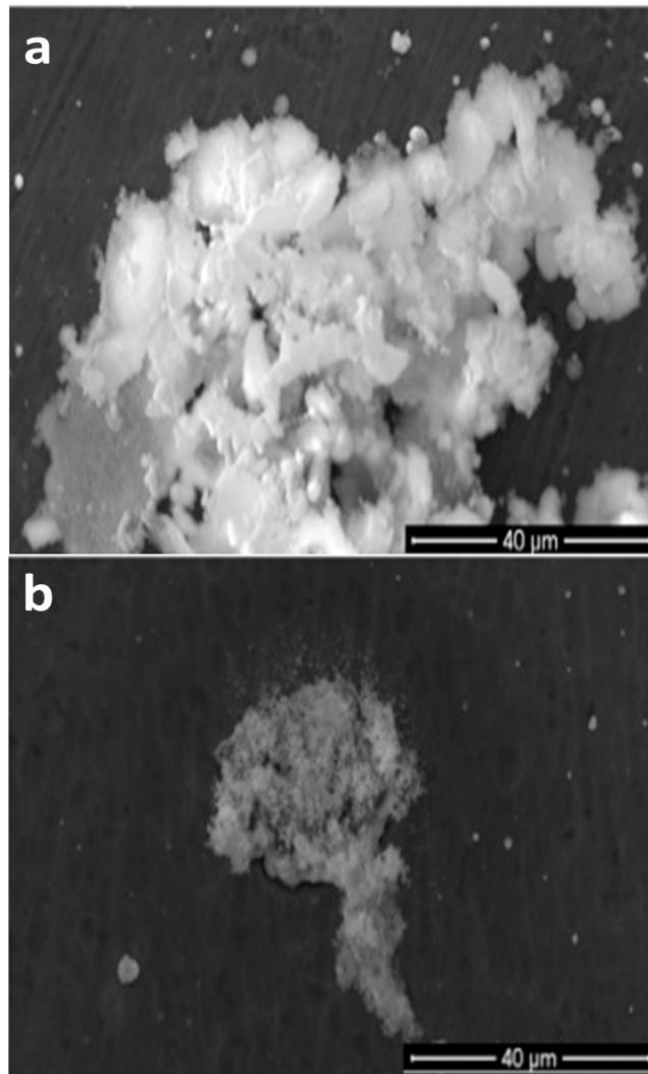


Figure 3.4 : SEM micrographs of HA deposits on a) TiN and b) (Ti,Mg)N after 4 weeks in 1X SBF (Onder et al., 2013).

3.2.2 XRD analysis

Although local HA deposits were observed in SEM micrographs of the surfaces, HA derived peaks (main peaks, 25.7° and 31.6°) that indicates the presence of HA deposits were not observed in the XRD patterns of both coatings after 4 weeks (Figure 3.5).

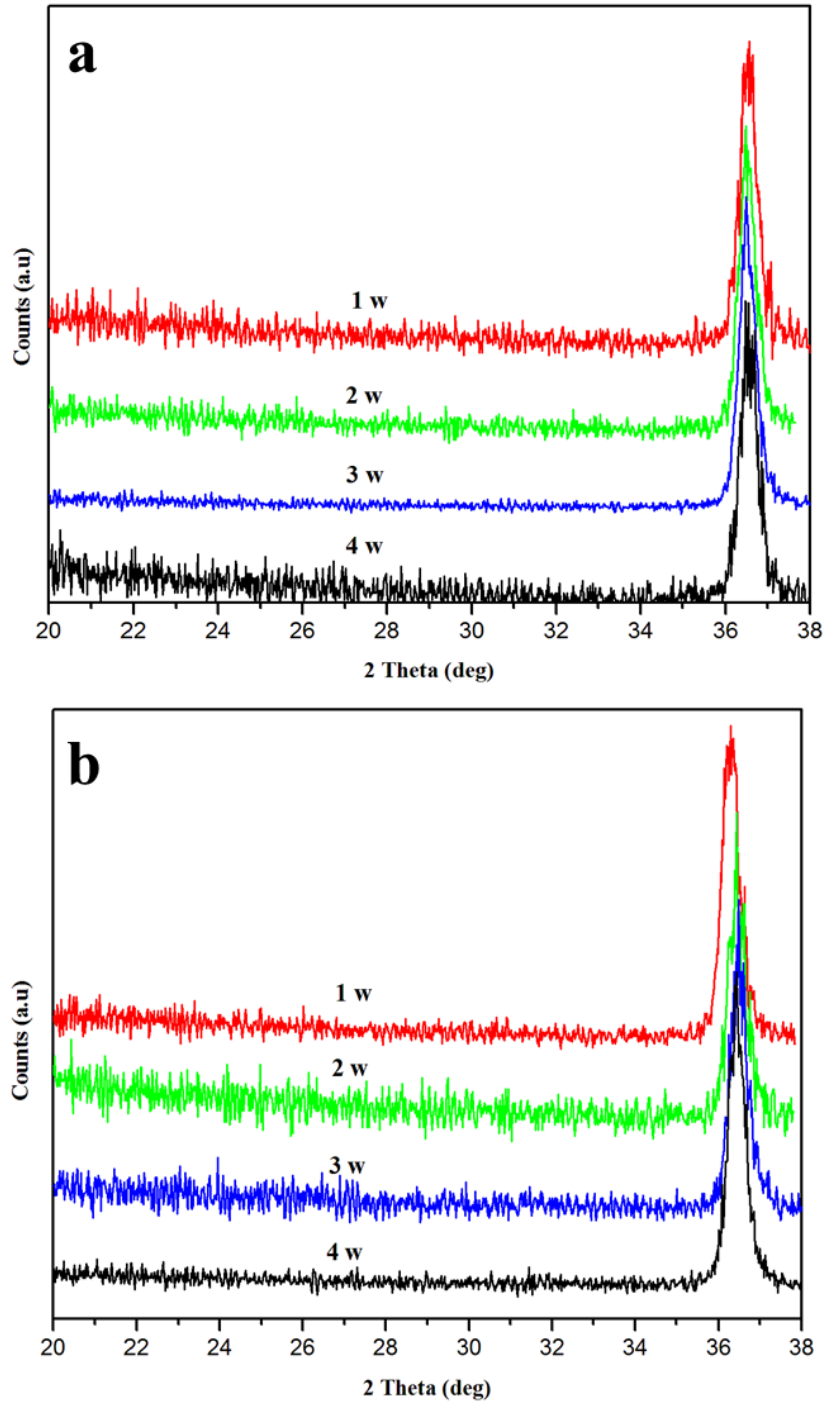


Figure 3.5 : XRD pattern of (a) TiN (b) (Ti,Mg)N substrates after 1XSBF incubation.

Nevertheless, the strongest (111) TiN peak (36.5°) of the (Ti,Mg)N coating shifted to the diffraction angle of magnesium free TiN (111) peak after 1st week of immersion (Figure 3.6). The shift of TiN peaks could be associated with the dissolution of Mg^{2+} from the (Ti,Mg)N structure. Mg^{2+} incorporation into TiN structure caused an increase in the lattice parameters of TiN structure. The calculated lattice parameters of the TiN and (Ti,Mg)N coatings were $a = 4.248 \text{ \AA}$ and $a = 4.253 \text{ \AA}$, respectively. Magnesium addition shifted the TiN peaks to lower diffraction angles (Figure 3.6) expanding the TiN lattice by 0.17 %. Hodroj et al. (2011) and Fenker et al. (2005) reported similar results showing that magnesium substitution for titanium atom in TiN structure resulted in lattice expansion of TiN phase because of higher atomic radius of magnesium.

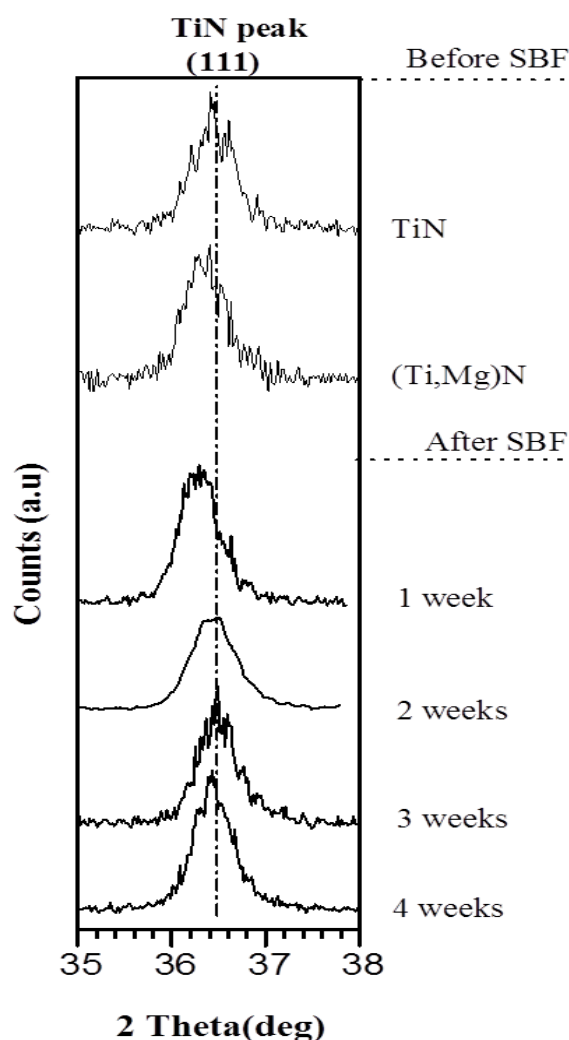


Figure 3.6 : XRD pattern of (Ti,Mg)N coatings after 1XSBF incubation (Onder et al., 2013).

3.2.3 FTIR analysis

FTIR analysis showed that HA deposition started after the first week on (Ti,Mg)N thin film coated surfaces. FTIR spectrum (Figure 3.7) of the (Ti,Mg)N sample exhibited PO_4^{3-} peaks, one of the characteristic HA peaks, around 553 and 1021 cm^{-1} . Although PO_4^{3-} peaks started to evolve after the second week in the spectrum of TiN, their intensity were not comparable to those of the (Ti,Mg)N even after 4 weeks.

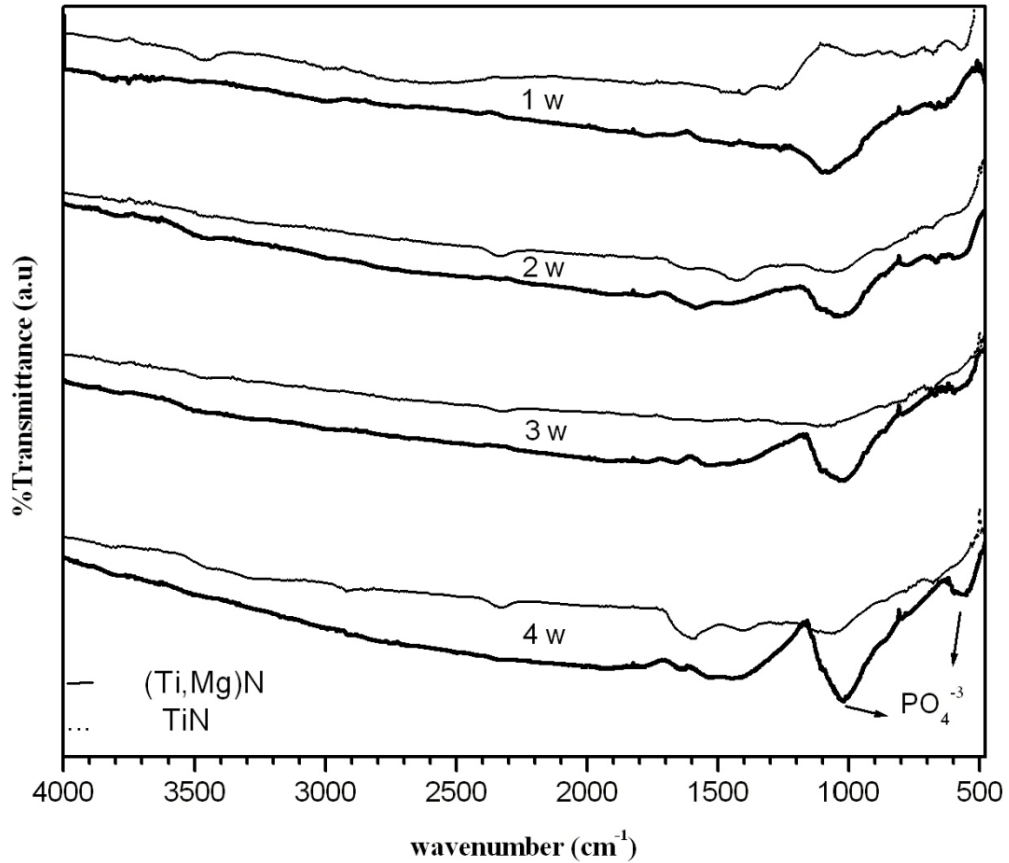


Figure 3.7 : FTIR spectra of deposits formed on TiN and (Ti,Mg)N coatings in 1XSBF (Onder et al., 2013).

3.3 Characterization of HA Deposits obtained in 5X SBF

Since the HA deposits formed in 1X SBF did not evenly cover the surfaces after 4 weeks, the HA formation experiments were repeated in 5X SBF solution for a duration of 3 days. There are many studies on which HA deposition was carried out in SBFs prepared at different concentrations (1-10 X) (Barrere et al., 2002; Bharati et al., 2005; Chou et al., 2004; Tas & Bhaduri, 2004). Depending on the SBF concentration, HA deposition time on substrates changes. For example, 1-3 weeks would be needed for HA precipitation on samples, when 1.5XSBF was used (Tas &

Bhaduri, 2004) . Nevertheless, 3 days would be enough to have HA deposits on substrates in 5XSBF. Temperature and pH values of prepared SBFs also affect the HA deposition, because the saturation rate of SBF is directly correlated with these parameters. Supersaturation is the key factor for HA deposition on substrates and HA start to deposit when the SBF reaches to supersaturation level (Koutsoukos et al., 2007). CO₂ release from SBF results in an increase in pH value of SBF that accelerates the supersaturation. Although CO₂ release does not cause a big difference in pH of 1XSBF (Tas & Bhaduri, 2004), it was found that 5XSBF with pH:7.4 reached to supersaturation level quickly. This was probably occurred because of more CO₂ release from 5XSBF. While HA deposits were observed at the bottom of the beakers, no HA deposition was found on samples. Therefore, in our study we set our pH value to 6.5 as Chou, et al., (2004).

3.3.1 SEM analysis

SEM micrographs showed that incubation of the samples in 5X SBF solution led to a complete coverage of both TiN and (Ti,Mg)N surfaces with HA deposits in 3 days (Figure 3.8). It could be clearly seen that the HA nodules on the (Ti,Mg)N surface developed to bigger sizes compared to those on the TiN surface because of the Mg²⁺ doping into HA structure (i.e. 2–3 times larger). Detailed SEM micrographs could be found in Figure D.2.

3.3.2 EDS analysis

HA structures produced on (Ti,Mg)N and TiN thin film coated surfaces were found to have different elemental compositions. Mg²⁺ presence in HA structures deposited on (Ti, Mg)N surfaces was clearly showed in analysis, while Mg²⁺ could not be detected in HA structures deposited on TiN. Ca/P ratios on HA structures deposited on (Ti,Mg)N and TiN were 1.87 and 1.91 respectively. Detailed elemental analysis of HA structures were given Table E.1 and Table E.2. Ca/P ratio changes depending on the HA type (Table 3.1). Ideal Ca/P ratio is about 1.67 in crystalline HA structure (Borodajenko, 2012). Our results indicated that Ca/P ratios of deposited HA structures on thin film coatings were close to ideal ratios but were not completely crystalline.

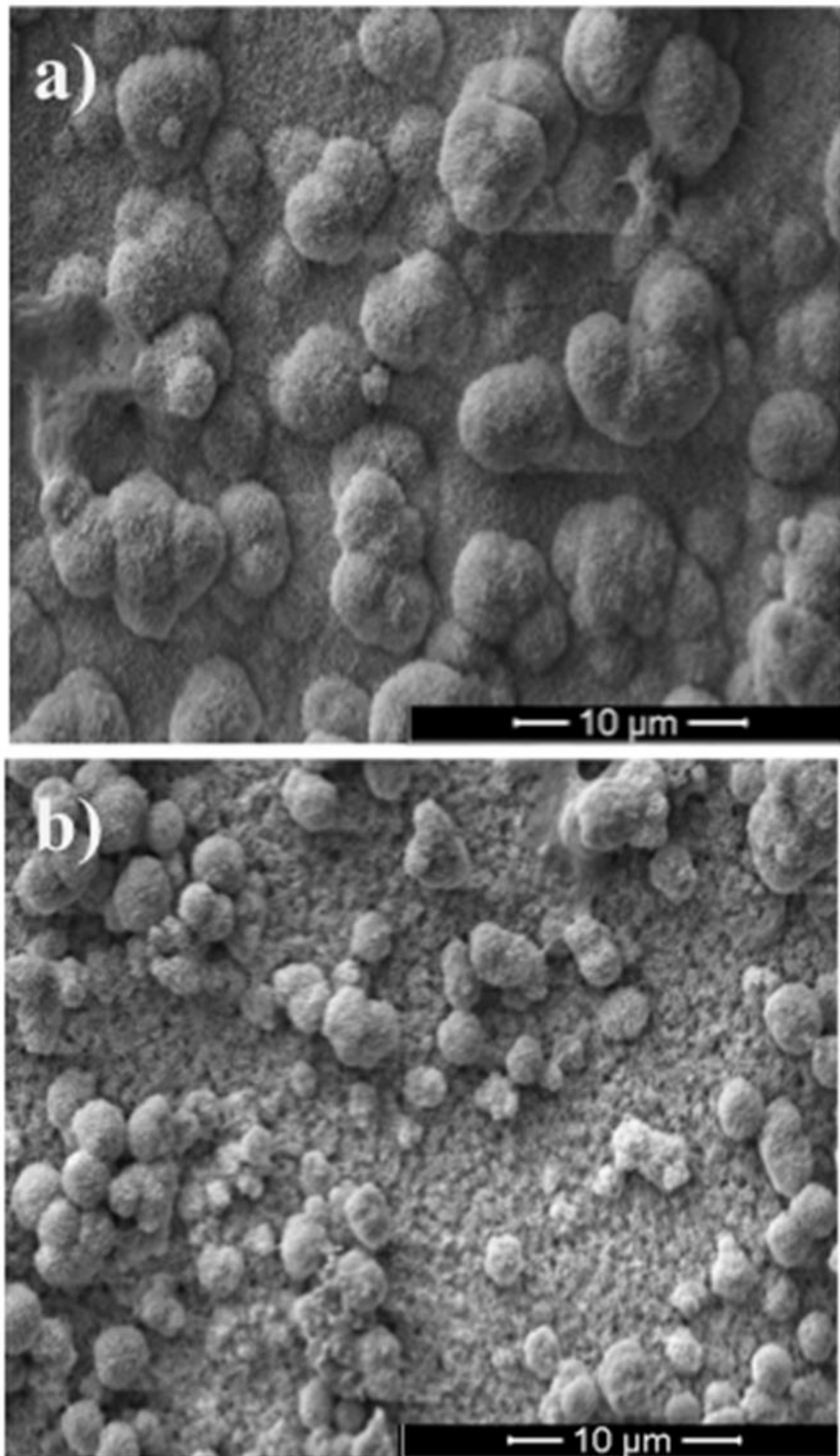


Figure 3.8 : SEM micrographs of (a) (Ti,Mg)N and (b) TiN coatings after 5X SBF incubation (Onder et al., 2013).

Table 3.1 Ca/P ratios in different HA deposits (Borodajenko, 2012).

Name	Abbreviation	Chemical formula	Ca/P
Amorphous calcium phosphate	ACP	$\text{Ca}_x\text{H}_y(\text{PO}_4)_z \cdot n\text{H}_2\text{O}$	1.2-2.2
Dicalcium phosphate anhydride	DCPA	CaHPO_4	1.00
Dicalcium phosphate dehydrate	DCPD	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	1.00
Octacalcium phosphate	OCP	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	1.33
β -tricalcium phosphate	β -TCP	$\text{Ca}_3(\text{PO}_4)_2$	1.50
α -tricalcium phosphate	α -TCP	$\text{Ca}_3(\text{PO}_4)_2$	1.50
Hydroxyapatite with calcium deficient	CDHA	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$ $0 \leq x \leq 1$	1.5-1.67
Hydroxyapatite	HAp	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	1.67
Tetra calcium phosphate	TTCP (TetCP)	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	2.00
β -Ca pyrophosphate	CPP	$\text{Ca}_2\text{P}_2\text{O}_7$	<1.5
Oxyapatite	OAp	$\text{Ca}_{10}(\text{PO}_4)_6\text{O}$	1.67

3.3.3 XRD analysis

The XRD patterns of the HA deposits on both coatings exhibited characteristic HA peaks after 5X SBF incubation. Characteristics HA peaks (about 25.7°, 31.6°, 28.2° and 39.6°) were detected on the (Ti,Mg)N surfaces (Figure 3.9). On TiN thin film coated surfaces, HA peaks were only obtained around 25.7° and 31.6°. Peak around 36.5° was also detected on both surfaces. This peak is a characteristic TiN peak and after 5XSBF incubation, it shifted to lower diffraction angles because of Mg^{2+} release like in the case of 1X SBF studies (Figure D.3).

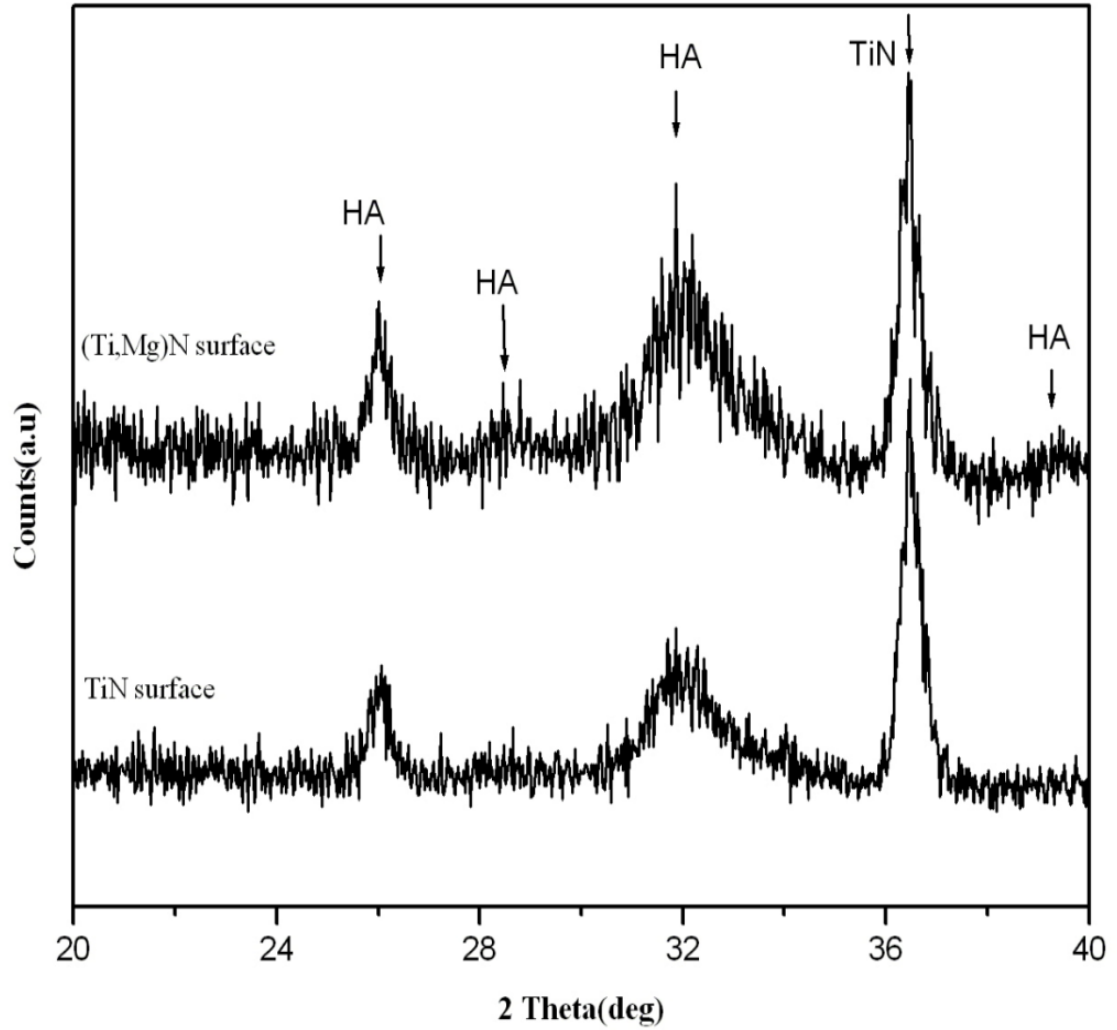


Figure 3.9 : XRD analysis of TiN and (Ti,Mg)N coatings after 5X SBF incubation (Onder et al., 2013).

3.3.4 FTIR analysis

FTIR spectra of the HA deposits on both coating were given in Figure 3.10. In the spectra, PO_4^{3-} group peaks at 475, 561, 600, 958, 1014 and 1115 cm^{-1} , HPO_4^{2-} group peak at 867 cm^{-1} , CO_3^{2-} peak at 1571 cm^{-1} and H_2O peak at 1634 cm^{-1} are characteristic peaks of a hydroxyapatite structure. These results were in accordance with the literature; Suchanek, et al., (2004) and Hanifi, et al., (2010) reported that PO_4^{3-} peaks became smaller with increasing amount of Mg in HA. Suchanek, et al., (2004) also reported that HPO_4^{2-} peak at 870 cm^{-1} evolved with higher magnesium contents in the HA. In our study, PO_4^{3-} peak at 475 cm^{-1} disappeared and CO_3^{2-} peaks at 1458 and 1571 cm^{-1} became more pronounced in the spectrum of the

(Ti,Mg)N sample (Figure 3.10a) when compared with the corresponding peaks of the TiN sample (Figure 3.10b).

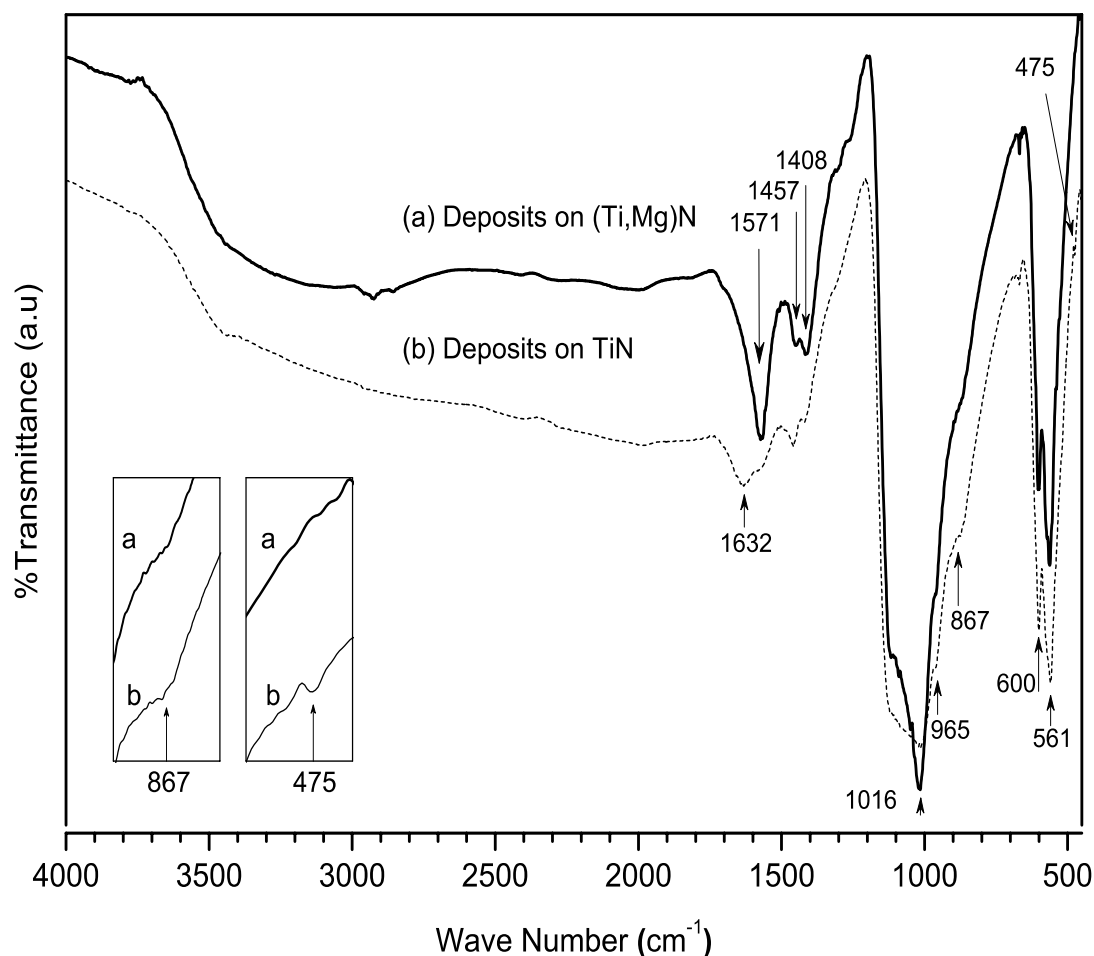


Figure 3.10 : FTIR spectra of (a) (Ti,Mg)N and (b) TiN coatings after 5X SBF incubation (Onder et al., 2013).

Hanifi, et al., (2010) and Suchanek, et al., (2004) also reported that substitution of CO_3^{2-} for PO_4^{3-} was particularly observed in the Mg substituted HA structure. The similarities of CO_3^{2-} , PO_4^{3-} and HPO_4^{2-} peaks with the existing literature (Hanifi et al., 2010; W. L. Suchanek et al., 2004) is a strong indication of the Mg^{2+} substituted HA formation on the (Ti,Mg)N coated sample. It could therefore be stated that the incorporation of Mg^{2+} into the TiN structure promoted the Mg substituted hydroxyapatite formation on the coating.

3.4 Characterization of HA Deposits Obtained in Mg-free 5X SBF

3.4.1 SEM analysis

For revealing the role of Mg^{2+} ions on the growth of HA for both coatings, the 5X SBF tests were repeated in the SBF without Mg^{2+} ions. After 3 days of incubation, there was no HA formation on the TiN sample (Figure 3.11b). Barrere, et al., (2002) showed the key role of Mg^{2+} ion on the formation of HA on Ti6Al4V substrates. They immersed the Ti6Al4V substrates in 5X SBF with and without Mg^{2+} ions. After 24 hours of immersion, they observed that HA deposits could only be formed in the solution containing Mg^{2+} ions. The results of our experiments further verified the importance of magnesium presence in solution during HA formation, this time in TiN coated samples.

It should, however, be noted that HA formation was observed on the surface of (Ti,Mg)N coating in 5X SBF solution even in the absence of Mg^{2+} ions (Figure 3.11a). This result showed the crucial role of Mg^{2+} incorporation on the thin films in the initiation of HA deposition.

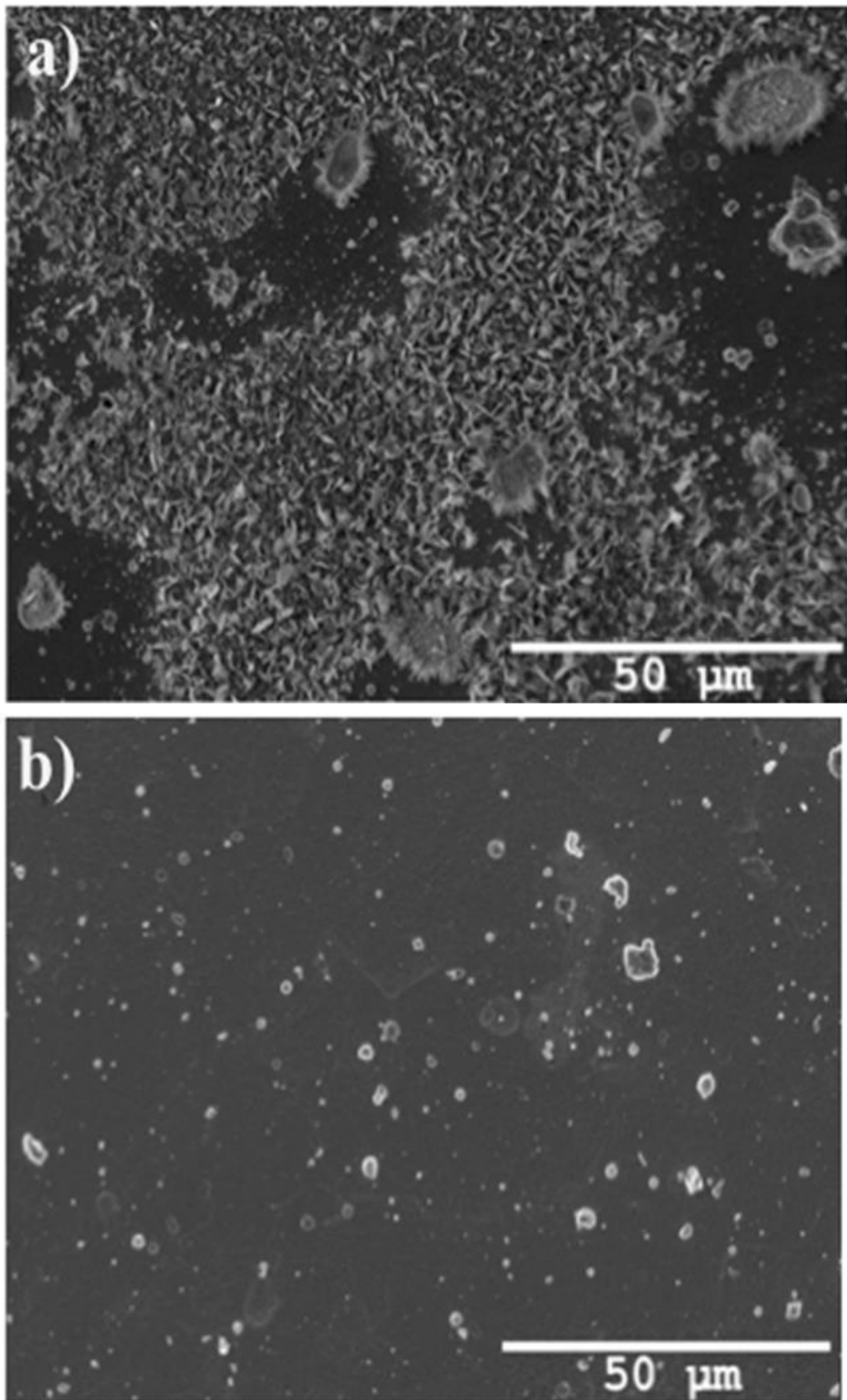


Figure 3.11 : SEM micrographs of (a) (Ti,Mg)N and (b) TiN coatings after Mg-free 5X SBF tests (Onder et al., 2013).

3.4.2 FTIR analysis

The HA derived peaks in the FTIR spectrum (Figure 12b) of the (Ti,Mg)N sample also showed the HA formation on the (Ti,Mg)N coating in this solution. All characteristic peaks of a crystalline hydroxyapatite structure, namely PO_4^{3-} group peaks at 475, 561, 600, 965, 1016 and 1115 cm^{-1} , HPO_4^{2-} group peak at 867 cm^{-1} , CO_3^{2-} peak at 1571 cm^{-1} and H_2O peak at 1634 cm^{-1} , could be detected. FTIR analysis also agree with the SEM results since FTIR peaks that indicated the HA deposition on the surfaces were not obtained on TiN surfaces (Figure 12a).

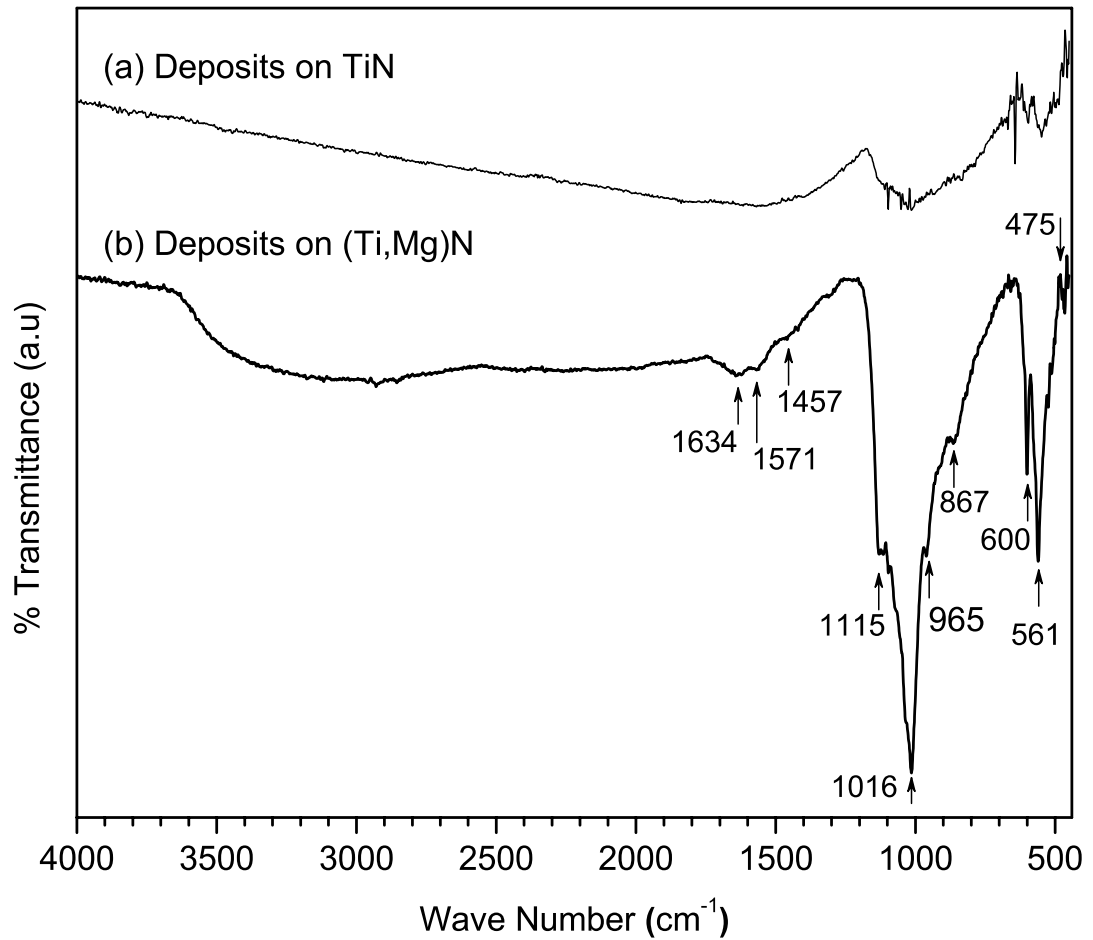


Figure 3.12 : FTIR spectra of (a) TiN and (b) (Ti,Mg)N coatings after Mg-free 5X SBF incubation (Onder et al., 2013).

These results clearly showed that the Mg in the (Ti,Mg)N coating promoted HA formation by releasing magnesium ions to the “SBF/coating” interface.

3.4.3 XRD analysis

Low density of characteristic HA peaks at about 25.7° and 31.6° were obtained on (Ti,Mg)N thin film coated surfaces after 3 days (Figure 3.13). Low-density peaks showed that crystallization process was not completed so poorly crystallized HA structures were deposited on the surfaces. In addition, peak around 36.5° indicating the TiN was also obtained on (Ti,Mg)N surfaces. On the other hand, the only peak obtained on TiN thin film coated surfaces was that of TiN, and any peak which would indicate HA deposition was not observed. It could therefore be said that XRD analysis further prove the positive contribution of Mg^{2+} on the HA deposition.

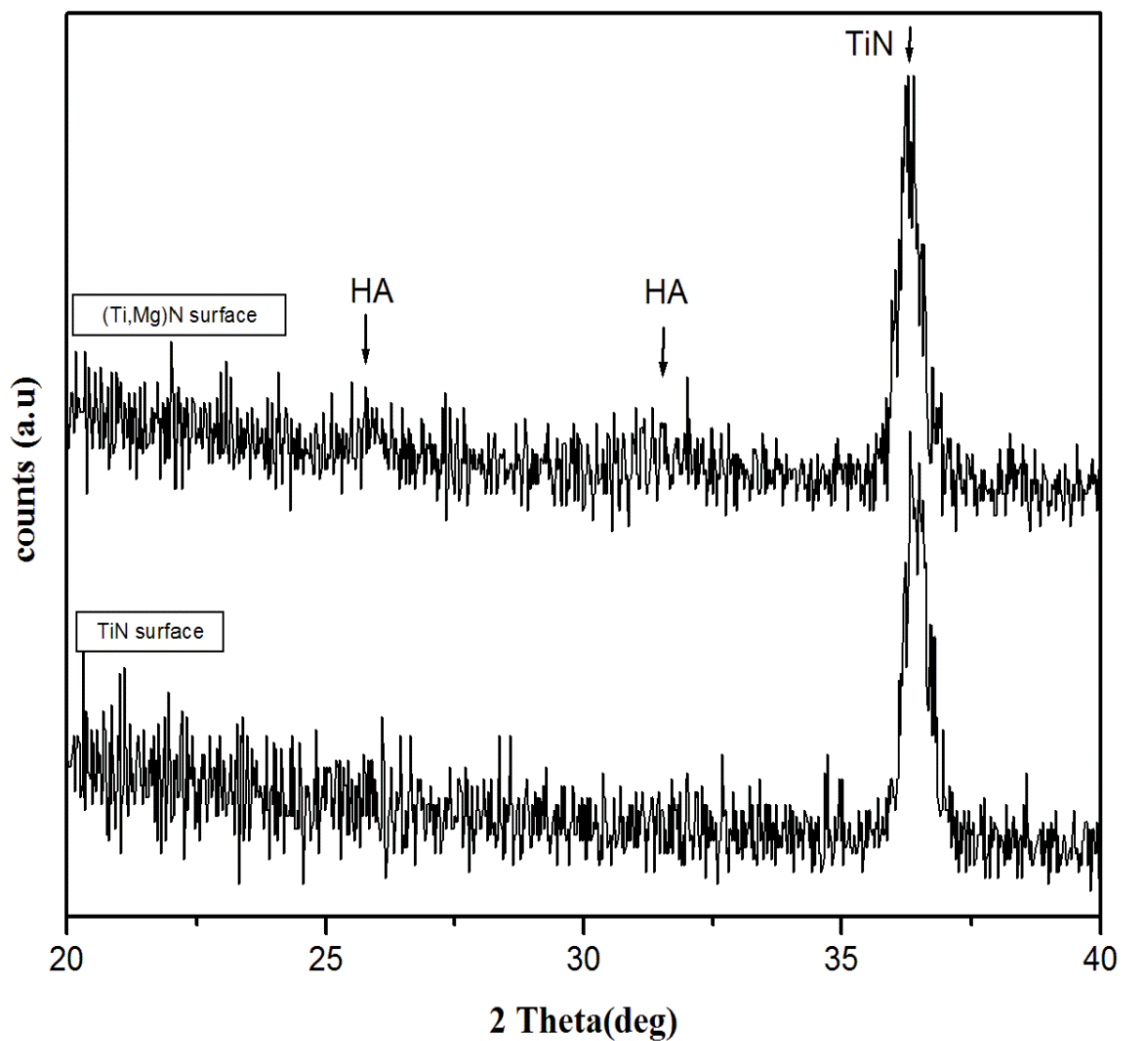


Figure 3.13 : XRD diffraction pattern of (Ti,Mg)N and TiN thin film coated surfaces after 5X Mg-free SBF studies.

3.5 Cell Proliferation on HA and Mg-HA Coated Surfaces Prepared in SBF

3.5.1 MTS calibration curve

R^2 value for the calibration curve was obtained as 0,9923 indicating a good linear fit (Figure 3.14). Equation obtained for linear fitting, $y = 5E-06x + 0,0867$, was used to determine the cell number after cell proliferation on HA and Mg-HA coated surfaces.

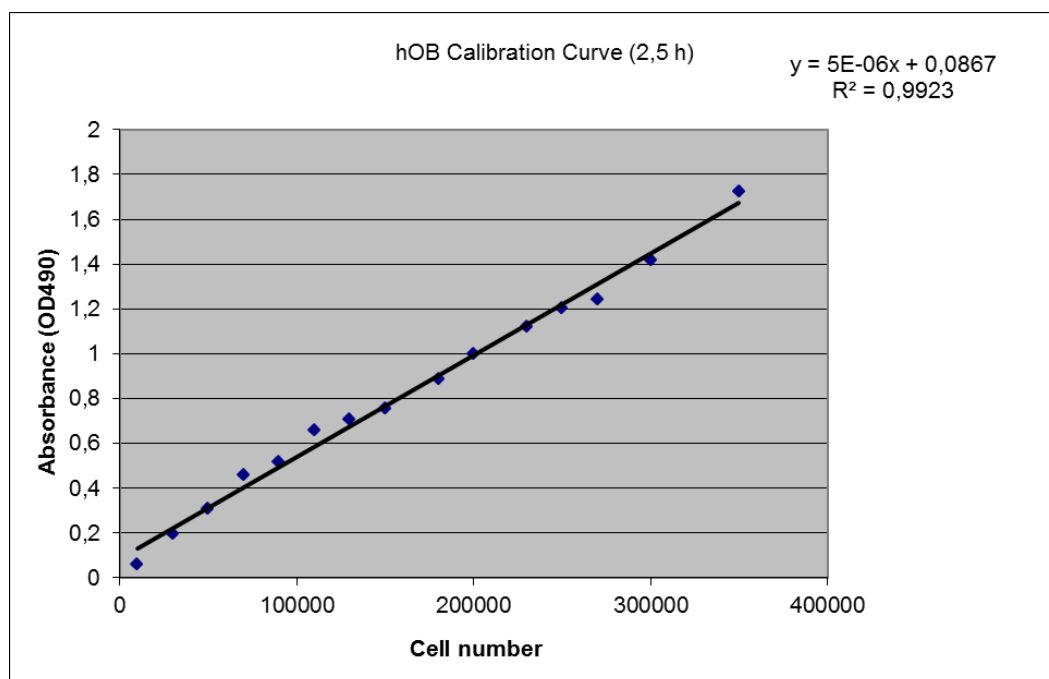


Figure 3.14 : hOB MTS calibration curve ($R^2 = 0,9923$).

3.5.2 MTS analysis

Cell proliferation studies showed that Mg-HA deposits on low Mg^{2+} (4.24 at %) containing (Ti,Mg)N surfaces are more suitable for cell proliferation than HA deposits on TiN surfaces (Mg^{2+} content 0 at %) (Figure 3.15). However, Mg-HA deposits synthesized on high Mg^{2+} (10.42 at %) containing surfaces was not suitable for cell proliferation, on the contrary it seemed like it discouraged the cell proliferation dramatically. Results are consistent with the literature since it is known that high amount of Mg^{2+} (> 5 mM) presence make toxic effect on cell proliferation as shown in MTS analysis (Leidi et al., 2011; Yun et al., 2010). Standart deviation results obtained for some samples could be originated from the effect of surface roughness on cell proliferation and cell detachment from the surfaces once the confluence was reached. Another factor affecting the results was the hydrophilicity of surfaces which was especially influencing initial cell attachment; when the cell solution was applied to the Ti surfaces, it was not always possible to keep all cells on

the surface and some of them were washed off and this might also lead to deviations in the results. .

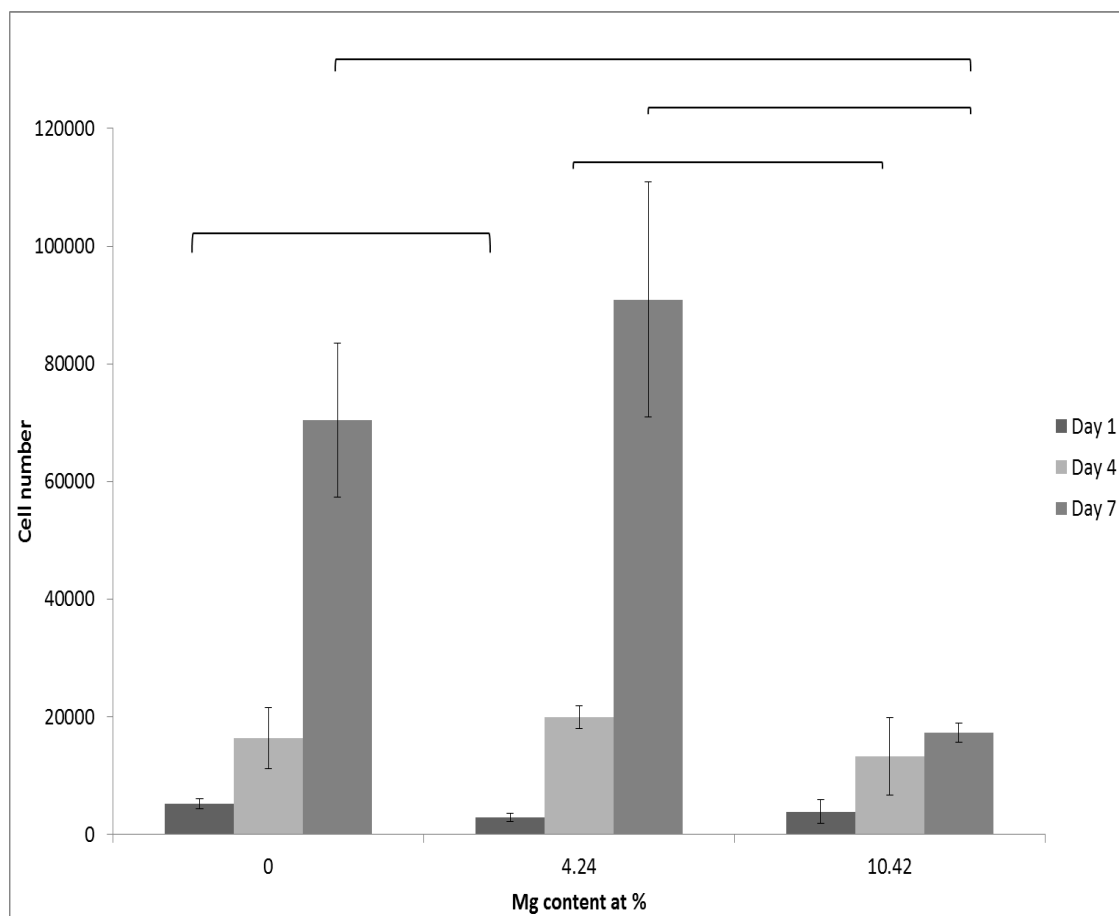


Figure 3.15 : Osteoblast cell proliferation on HA and Mg-HA deposits. Significant differences were labeled on the graph for $p \leq 0.05$ ($n=3$).

3.5.3 SEM analysis

SEM micrographs were also supported the MTS results since number of cells on the Mg-HA synthesized on low Mg^{2+} (4.24 at %) doped (Ti,Mg)N thin film coated surfaces (Figure 3.16b) was significantly higher than HA coated TiN surfaces (Figure 3.16a). Furthermore, osteoblasts seem healthier on the HA and Mg-HA synthesized on low Mg^{2+} (4.24 at %) containing (Ti,Mg)N thin film coated surfaces, that is, their distribution and proliferation were better on these surfaces compared with the Mg-HA coated surfaces that were deposited on 10.42 at % Mg^{2+} containing thin film coatings. When the Mg^{2+} content increased in the HA structure, osteoblast morphology changes (Figure 3.16c). They did not expand and did not make contact with other cells like healthy cells did.

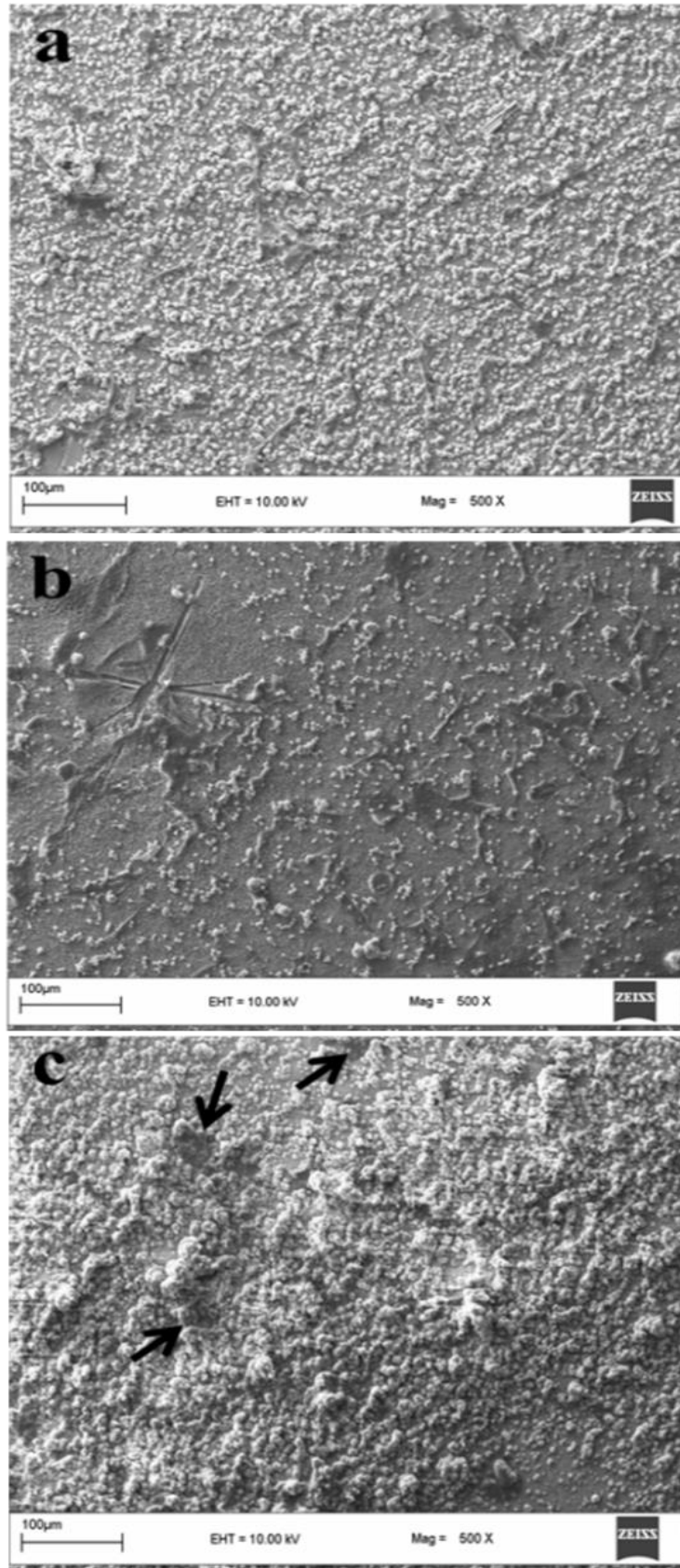


Figure 3.16 : SEM micrographs of osteoblast cells on a) HA, b) Mg-HA deposits on low Mg^{2+} (4.24 at %) containing and c) Mg-HA deposits on high Mg^{2+} (10.42 at %) containing surfaces.

3.5.4 Fluorescence microscopy analysis

Fluorescence microscopy analysis showed that there was no detectable change in morphology of osteoblast cells whether they proliferate on HA or Mg-HA coated surfaces (Figure 3.17). However, cell density on Mg-HA coatings that were deposited on low Mg^{2+} containing surfaces was the highest which was in agreement with the MTS results (Figure 3.17b). Moreover, change in morphology of osteoblasts in high Mg^{2+} ratio observed in SEM analysis was confirmed in fluorescence microscopy analysis (Figure 3.17c). Similar type of crouched cells with a limited number of extended arms were visualized under the microscope.

3.6 Cell Culture Studies on TiN and (Ti,Mg)N Thin Film Coatings with Various Mg^{2+} Amounts

3.6.1 TiN and (Ti,Mg)N thin film coatings with various Mg^{2+} amounts

(Ti,Mg)N coatings with various Mg^{2+} amount were given in Table 3.2. These thin film coated substrates were used to determine the effect of Mg^{2+} on both cell proliferation and corrosion behavior of substrates.

Table 3.2: (Ti,Mg)N coatings with different Mg^{2+} contents.

Mg^{2+} amount (at %)	Ti (1) Cathode (A)	Mg (2) Cathode (A)	Ti (3) Cathode (A)
0	75	0	70
8.1	80-85	15-20	85
11.31	80	25-30	80
28.49	75	45-50	70

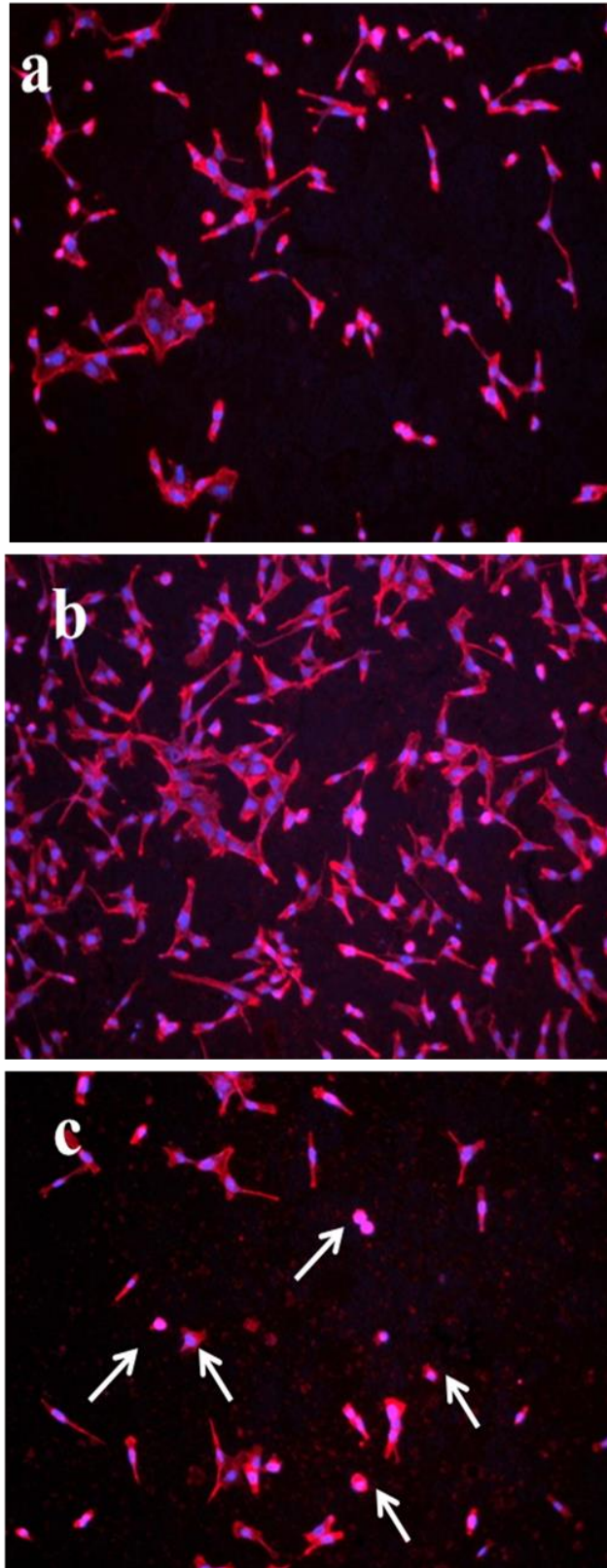


Figure 3.17 : Fluorescence images of osteoblast cells after staining with Phalloidin and DAPI on (a), HA and on Mg-HA coatings deposited (b) 4.24 at % and (c) 10.42 at % Mg^{2+} containing surfaces after 4 days.

3.6.2 Cell proliferation on bare and Mg²⁺ doped TiN thin film coatings

3.6.2.1 MTS analysis

MTS analysis showed that Mg²⁺ doping into TiN thin films affect the cell proliferation in a concentration dependent manner (Figure 3.18). Cell proliferation on 8.1 at % Mg²⁺ containing (Ti,Mg)N thin film coated surfaces was better compared with the bare TiN thin film coated surfaces. However, cell proliferation significantly decreased when higher Mg²⁺ amounts (11.31 and 28.49 at %) were used. Mg²⁺ is a cofactor for many enzymes some of which are known to induce the proliferation of osteoblast cells, and while low amount of Mg²⁺ make an insulin effect on cell proliferation, high amount of Mg²⁺ exhibit toxic effect hindering the cell growth (Takaya et al., 2004). Toxicity due to the high Mg²⁺ amount might therefore be one of the possible reasons for the slowdown in cell proliferation. Besides this, our group showed that Mg²⁺ amount in the (Ti,Mg)N thin film coatings should be under 10 at% to have a better corrosion resistance in a previous study (Demirel, 2003). Accelerated corrosion of (Ti,Mg)N thin films may therefore be the another reason why cell proliferation decreased with the increasing Mg²⁺ amount in the thin film coatings. As corrosion affects the cell attachment on the surface, cell proliferation could also be affected and decreased.

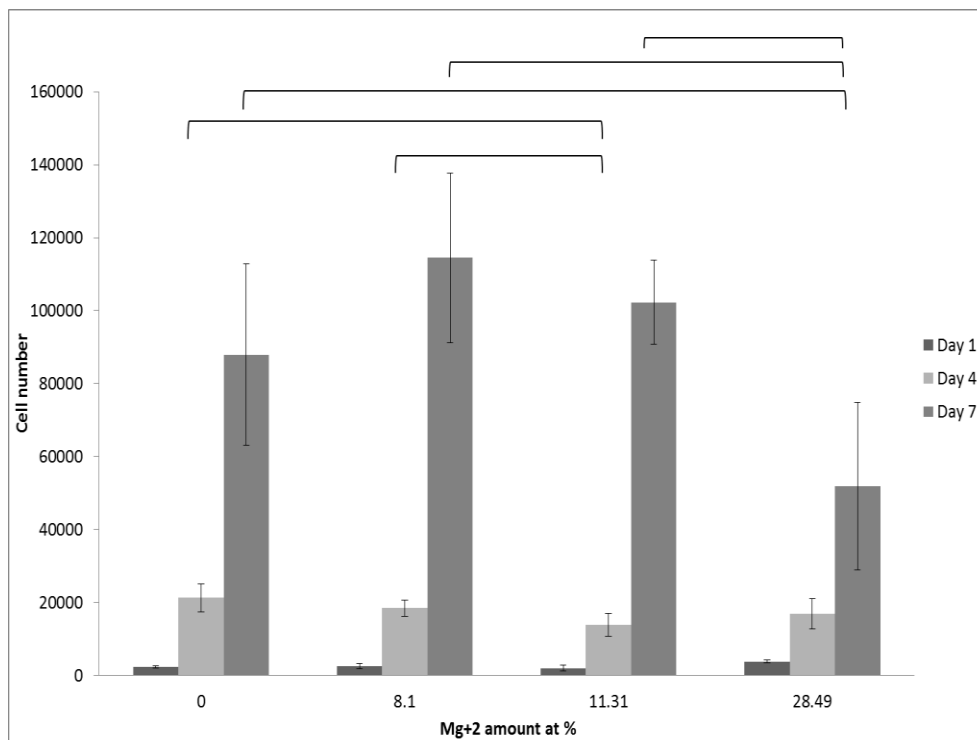


Figure 3.18 : Effect of Mg²⁺ on cell proliferation. Statistically significant differences were labeled on the graph for p≤0.05 (n=3)

3.6.2.2 SEM analysis

SEM micrographs taken after a week of incubation also supported the MTS results since number of cells on the low (8.1 at %) Mg^{2+} doped (Ti,Mg)N thin film coated surfaces was higher than TiN surfaces (Figure 3.19). Furthermore, osteoblast cell distribution and spreading were better on the low (8.1 at %) Mg^{2+} containing (Ti,Mg)N surfaces (Figure 3.19b) compared with the TiN ones (Figure 3.19a). Change in morphology of cells depending on the Mg^{2+} amount was also observed in SEM micrographs. When Mg^{2+} content exceeded 10 at %, the cell morphology changed (Figure 3.19c and Figure 3.19d) which was also a clue for negative effect of high Mg^{2+} on the osteoblast cell. The same situation was also observed in cell culture studies made on Mg-HA structures deposited on high (more than 10 at %) Mg^{2+} containing (Ti,Mg)N surfaces (Figure 3.16c).

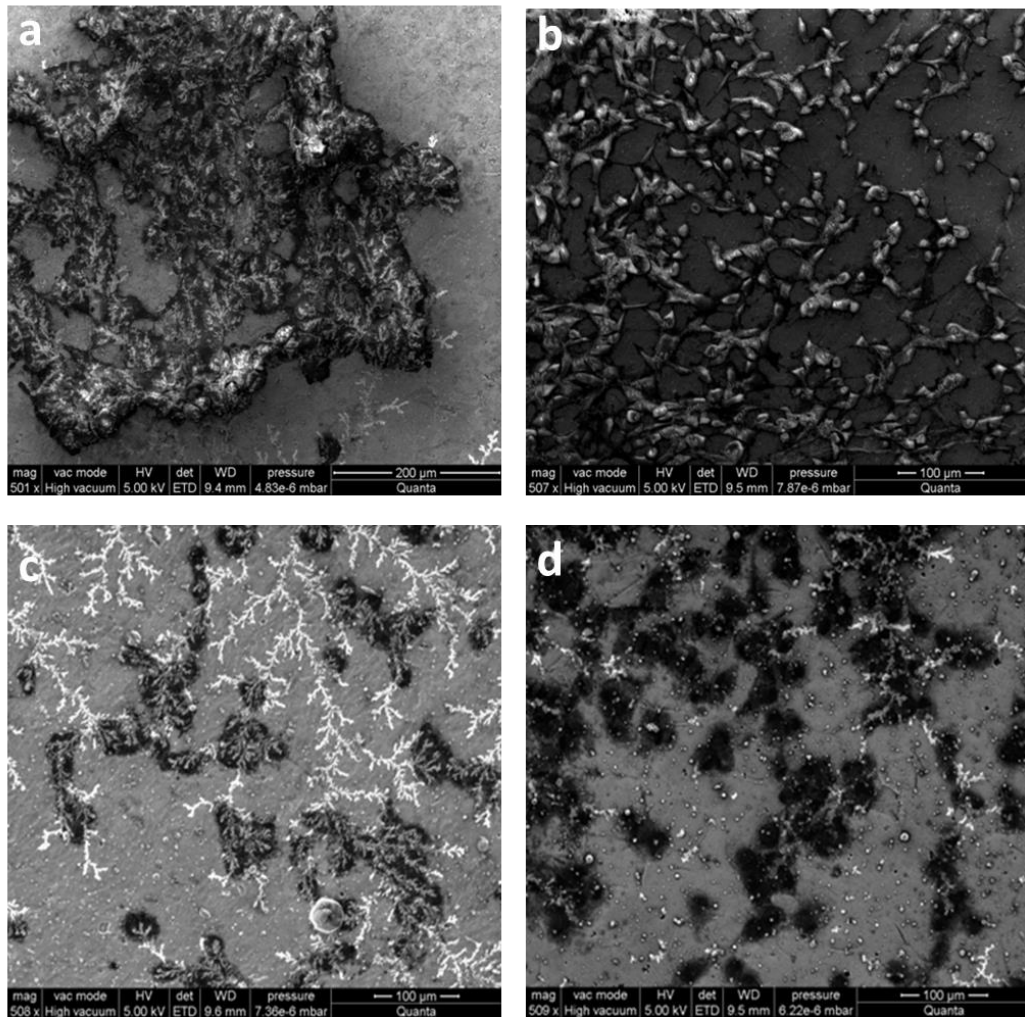


Figure 3.19 : Osteoblast cells on a) TiN, b) 8.1 at % Mg^{2+} , c) 11.31 at% Mg^{2+} and d) 28.49 at % Mg^{2+} containing (Ti,Mg)N thin film coatings after 7 days.

3.6.2.3 Fluorescence microscopy analysis

Fluorescence microscopy analysis (Figure 3.20) showed that there was no detectable change in morphology of osteoblast cells whether they proliferate on TiN or Mg²⁺ doped TiN thin film coated surfaces.

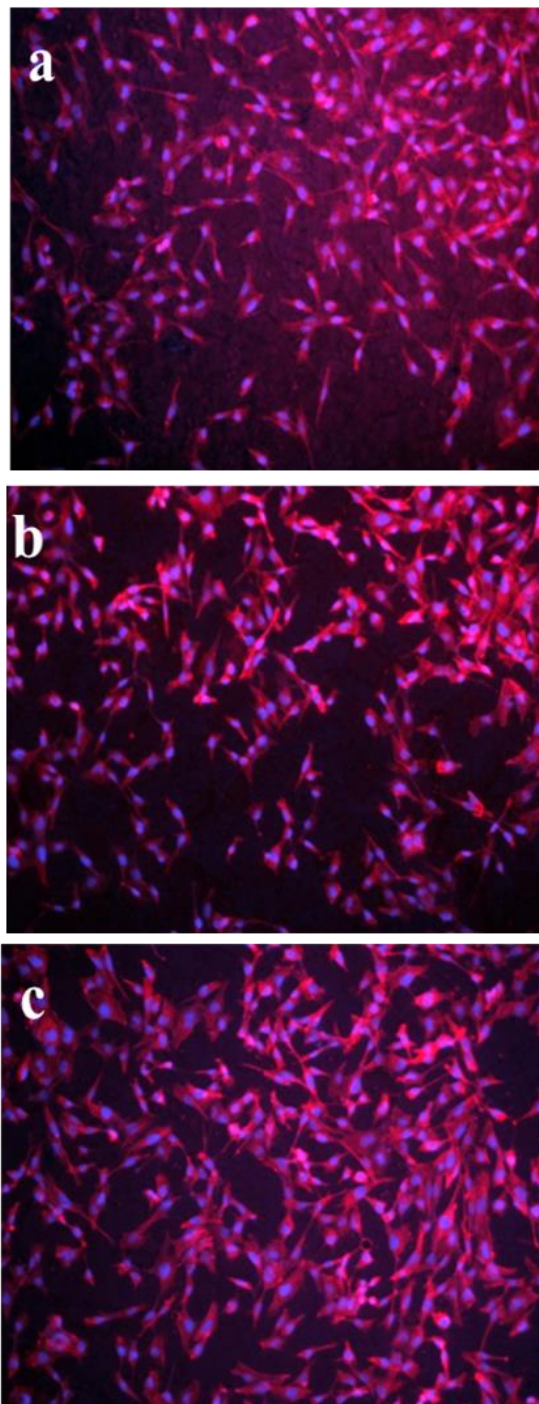


Figure 3.20 : Fluorescence images of osteoblast cells after staining with Phalloidin and DAPI on (a) TiN (b) 8.1 at % and (c) 11.31 at % Mg²⁺ doped TiN surfaces after 7 days.

3.7 Hydroxyapatite Deposition and Corrosion in Cell Culture Medium

3.7.1 SEM analysis

SEM micrographs showed that while HA nucleation (dark fields on the surfaces) started on TiN after the first week (Figure 3.21a), it was delayed on Mg^{2+} containing (8.1, 11.31 and 28.49 at %) (Ti,Mg)N thin film coated surfaces. HA nucleation durations on these coatings considerably changed with the Mg^{2+} amounts. HA nucleation (dark fields) on low Mg^{2+} (8.1 at %) containing (Ti,Mg)N surfaces were observable after 14 days (Figure 3.21b), whereas no detectable HA nucleation was detected on high Mg^{2+} containing surfaces after 14 days (Figure 3.21c,d).

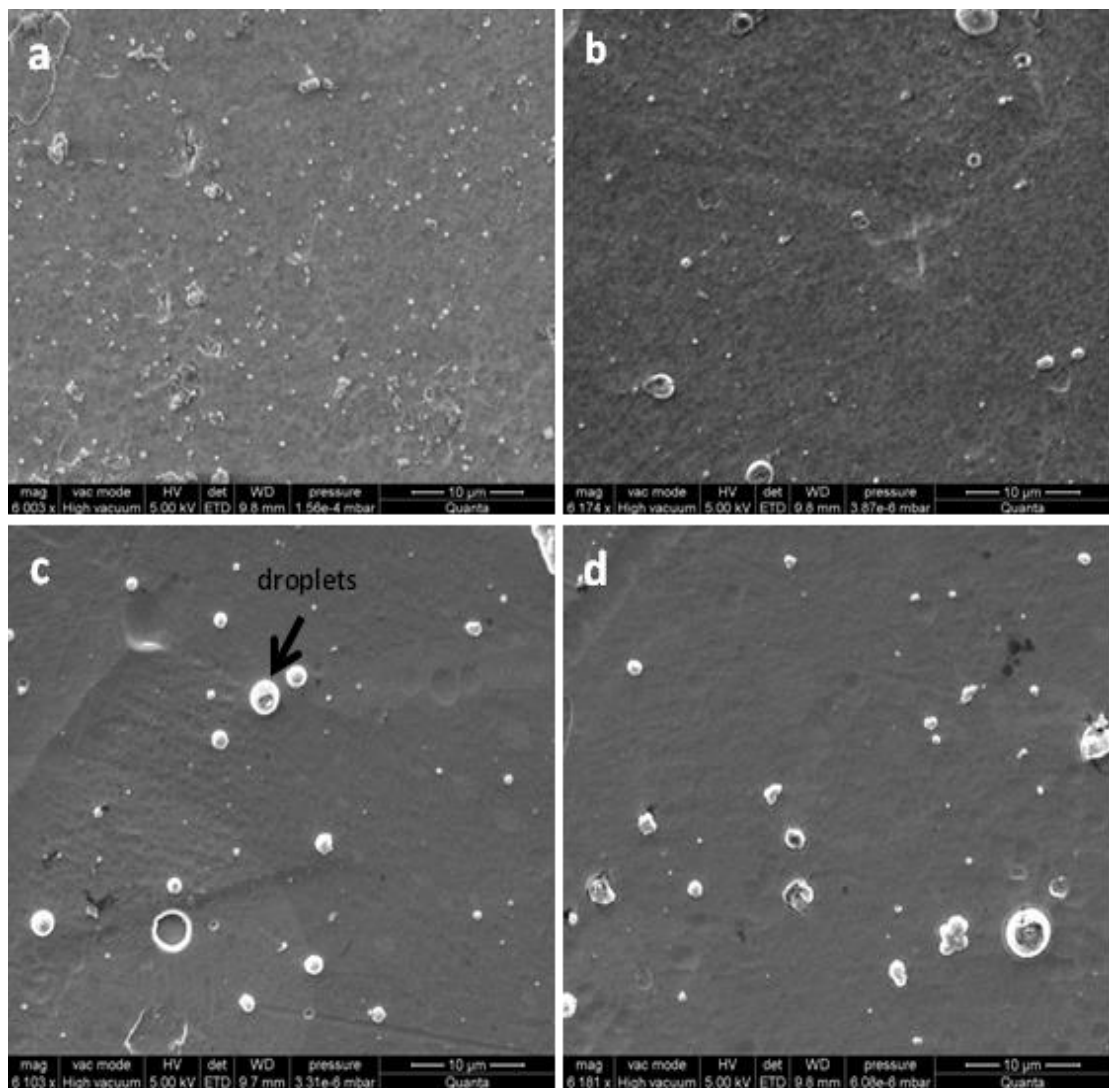


Figure 3.21 : HA nucleation on a) TiN after 7 days b) 8.1 at % Mg^{2+} doped c) 11.31 at % Mg^{2+} and d) 28.49 at % Mg^{2+} doped TiN surfaces after 14 days.

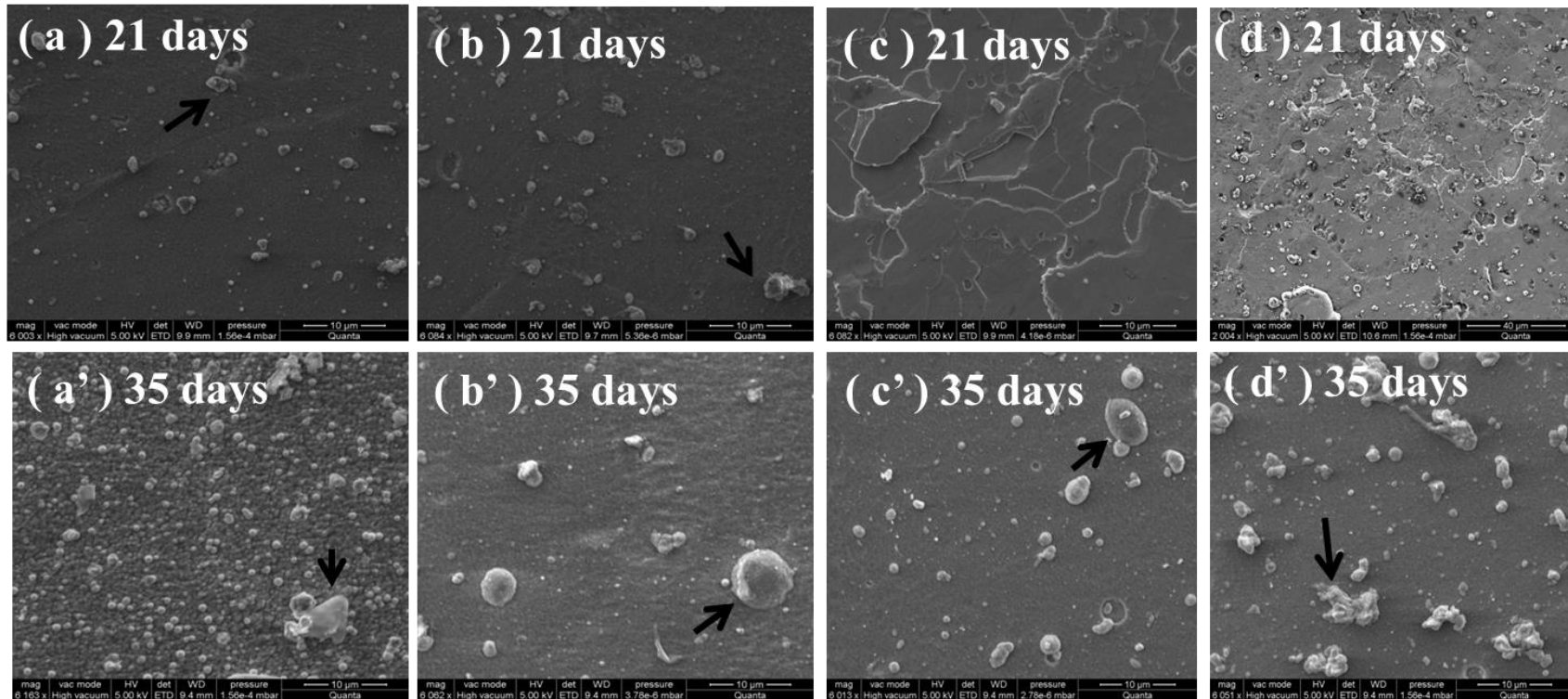
Besides this, granule size of HA deposits was smaller on (Ti,Mg)N thin film coated surfaces compared with deposits on TiN thin film coated surfaces. Size difference of

Besides this, granule size of HA deposits was smaller on (Ti,Mg)N thin film coated surfaces compared with deposits on TiN thin film coated surfaces. Size difference of HA deposits seemed to be more pronounced on TiN surfaces after the 35 days (Figure 3.22a', b', c' and d'). Additional SEM micrographs of thin film coatings could be found for 42,49 and 56 days in Figure D.4

On the other hand, corrosion was observed on 11.31 and 28.49 at % Mg^{2+} containing (Ti,Mg)N thin film coated surfaces after 21 days (Figure 3.22c,d). It could therefore be said that in order to have a better corrosion resistance, Mg^{2+} amount should be set under 10 at % which was in consistency with the previous results (Demirel, 2003). New HA deposits were also observed on the corroded surfaces (Figure 3.22c',d'). No detectable corrosion could be seen on TiN (Figure 3.22a) or 8.1 at % Mg^{2+} (Ti,Mg)N (Figure 3.22b) thin film coated surfaces after 21 days. All these findings indicated that corrosion resistance, HA deposition time, and granule size were highly depended on the Mg^{2+} amount in the thin film coatings.

3.7.2 EDS analysis

EDS analysis were done to determine the Ca/P ratios on the TiN and (Ti,Mg)N thin film coatings after HA deposition studies. It was possible to determine Ca/P ratio on TiN and low Mg^{2+} (8.1 at %) containing (Ti,Mg)N after 7 days, but Ca/P ratio can only be determined after 14-21 days on high Mg^{2+} containing (11.31 and 28.49 at %) surfaces. HA deposition on these surfaces was probably delayed because of Mg^{2+} release from the thin film coatings. Detailed EDS analysis could be found in Table E.3.



arrows points out Ti-Mg droplets, not HA granules

Figure 3.22 : HA deposits and corrosion on the (a,a') TiN , (b,b') 8.1 at % Mg^{2+} , (c,c') 11.31 at % Mg^{2+} and (d,d') 28.49 at % Mg^{2+} containing (Ti,Mg)N thin film coated surfaces.

3.7.3 XRD analysis

HA structures were more obvious on thin film coated surfaces after 35 days. Therefore, in order to determine HA deposition and its crystal structure on the surfaces, samples that were incubated for 35 days in cell culture medium in the absence of cells were chosen for XRD analysis. While TiN peaks around 36.27° , were obtained in all samples, no HA peak obtained in any surfaces (Figure 3.23) which could be the result of a thin layer of HA deposit or a amorphous structure.

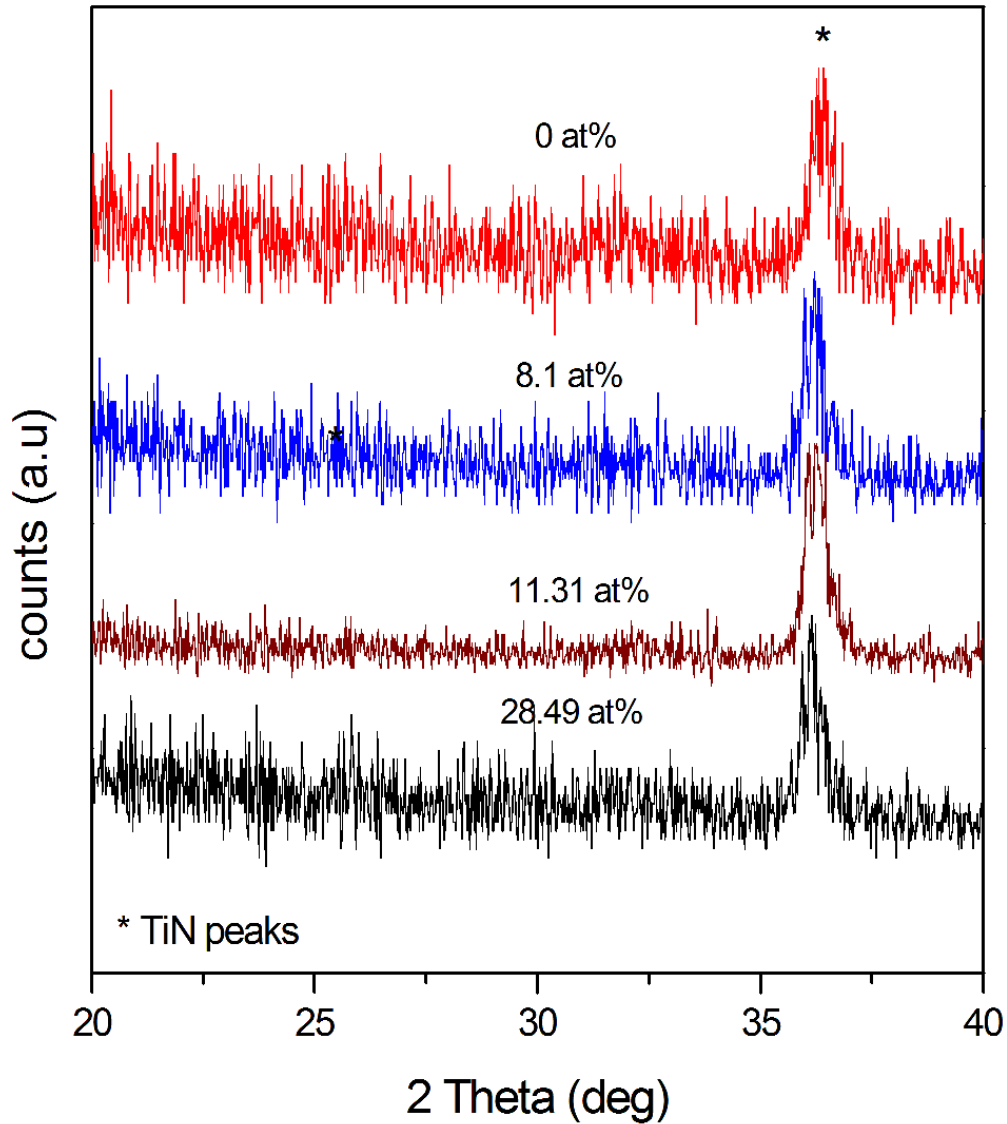


Figure 3.23 : XRD diffraction pattern of (Ti,Mg)N and TiN thin film coated surfaces after incubation in cell culture medium (35 days).

3.7.4 FTIR analysis

FTIR analysis also supported SEM and EDS results; Characteristic HA peaks were obtained on the TiN after 7 days (Figure 3.24a). However, amide peaks (1660, 1555 and 1280 cm^{-1}) were obtained on 8.1, 11.31 and 28.49 at % Mg^{2+} doped TiN surfaces after 7 days which indicated that Mg^{2+} doping on to the surfaces resulted in protein absorption on surfaces. While high intensity PO_4^{3-} peaks obtained around 610 and 1077 cm^{-1} on TiN surfaces, only low intensity PO_4^{3-} peaks were obtained on 8.1 at % Mg^{2+} containing (Ti,Mg)N around 1077 cm^{-1} . After 28 days incubation time, crystalline HA peaks (CO_3^{2-} , 1429 cm^{-1} ; PO_4^{3-} , 577, 607 and 1101 cm^{-1} ; -OH, 1652 cm^{-1} ; H_2O , 3261 cm^{-1}) on both TiN and low Mg^{2+} (8.1 at %) containing surfaces were started to be detected (Figure 3.24b). HA deposits on high Mg^{2+} (11.31 and 28.49 at %) doped thin film coatings were poorly crystallized compared with the TiN or low Mg^{2+} doped surfaces. However, HA deposits on these surfaces also turned into a more crystalline form after 42 days (Figure 3.24c). PO_4^{3-} peaks around 587, 609 and 1114 cm^{-1} , CO_3^{2-} peaks around 1430 cm^{-1} , -OH peaks around 1653 cm^{-1} and absorbed water peaks around 3323 cm^{-1} were obtained on all surfaces. HPO_4^{2-} peaks around 878 cm^{-1} showed that there was still calcium deficiency in HA deposits.

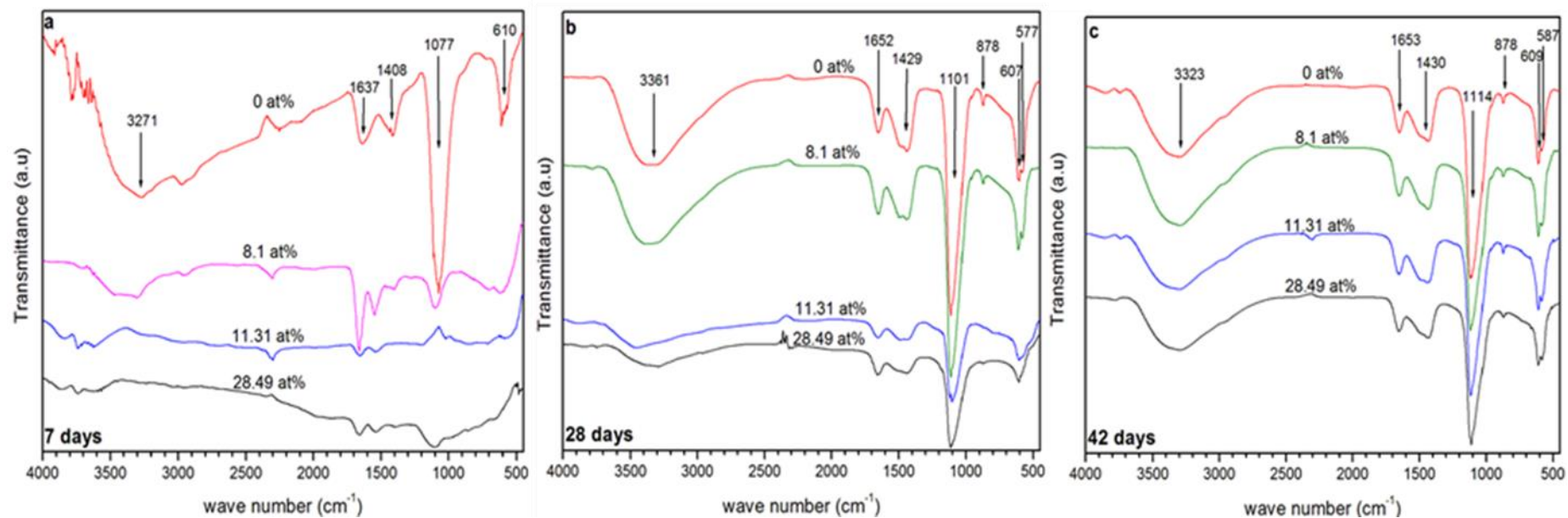


Figure 3.24 : FTIR analysis made on TiN and (Ti,Mg)N thin film coated surfaces after cell culture medium.

3.8 Hydroxyapatite Deposition and Corrosion in the Presence of Cells

3.8.1. SEM analysis

SEM micrographs of the cell seeded surfaces showed that when the osteoblasts on thin film coated surfaces reached to the confluence (Figure 3.25a), they started to detach and leave a surface with low cell density (Figure 3.25b). Then, cells start to repropagate on the newly opened places on the surface (Figure 3.25c).

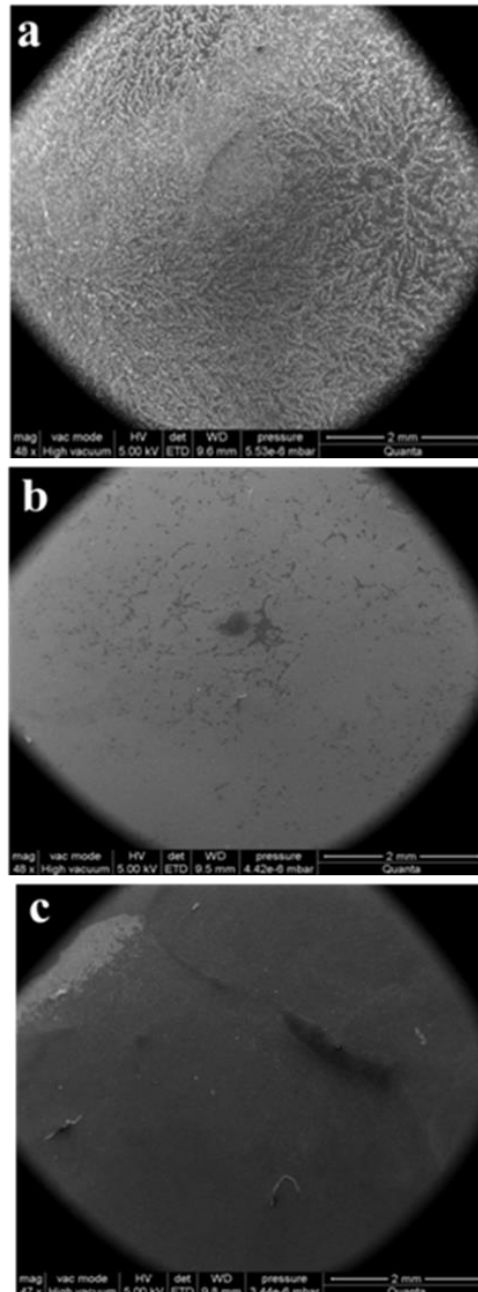


Figure 3.25 : Osteoblast cell separation on 8.1 at % Mg²⁺ doped TiN surfaces a) 7days b) 14 days c) 21 days.

The cell number have therefore been fluctuating throughout the HA deposition process. SEM micrographs also showed that mineralized structures increased after the first week on TiN (Figure 3.26a) and after second week on (Ti,Mg)N thin film coated surfaces (Figure 3.26b, c, and d) and their thickness increased with time (Figure 3.26).

3.8.2. EDS analysis

Ca/P ratio on the thin film coated surfaces is an important clue for the hydroxyapatite deposition. EDS analysis showed that Ca/P ratio decreased and increased periodically over a period of 56 days (Figure 3.27). The cycle probably occurred due to the dislocation of mineralized structure from the surfaces when it reached to a particular thickness or when the cells detached from the surface.

Moreover, mineralized structure might dissolve from the thin film coated surfaces because of pH changes so Ca/P ratio may change depending on the dissolution rate. Ideal Ca/P ratio in a crystalized HA structure should be 1.67 (Borodajenko, 2012) and reaching this value on high Mg^{2+} containing (Ti,Mg)N surfaces (11.31 and 28.49 at %) took more time compared with the Mg^{2+} free and low Mg^{2+} (8.1 at %) containing surfaces probably because of the high dissolution rate of Mg^{2+} ions. While Ca/P ratio on TiN and low Mg^{2+} containing (Ti,Mg)N surfaces were nearly ideal after 14 days, the ratio was very low (ca. 0.2) on high Mg^{2+} containing surfaces. It was also found that Ca/P ratio decreased with the increasing Mg^{2+} amount in the coatings after 21 days. After 35 days, however, all surfaces showed a similar behavior and almost ideal Ca/P ratios on all surfaces obtained between 35-42 days. Then, another decrease after 42 days and an increase after 49 days on Ca/P ratios was observed on all surfaces. Detailed elemental analysis was given in Table E.3.

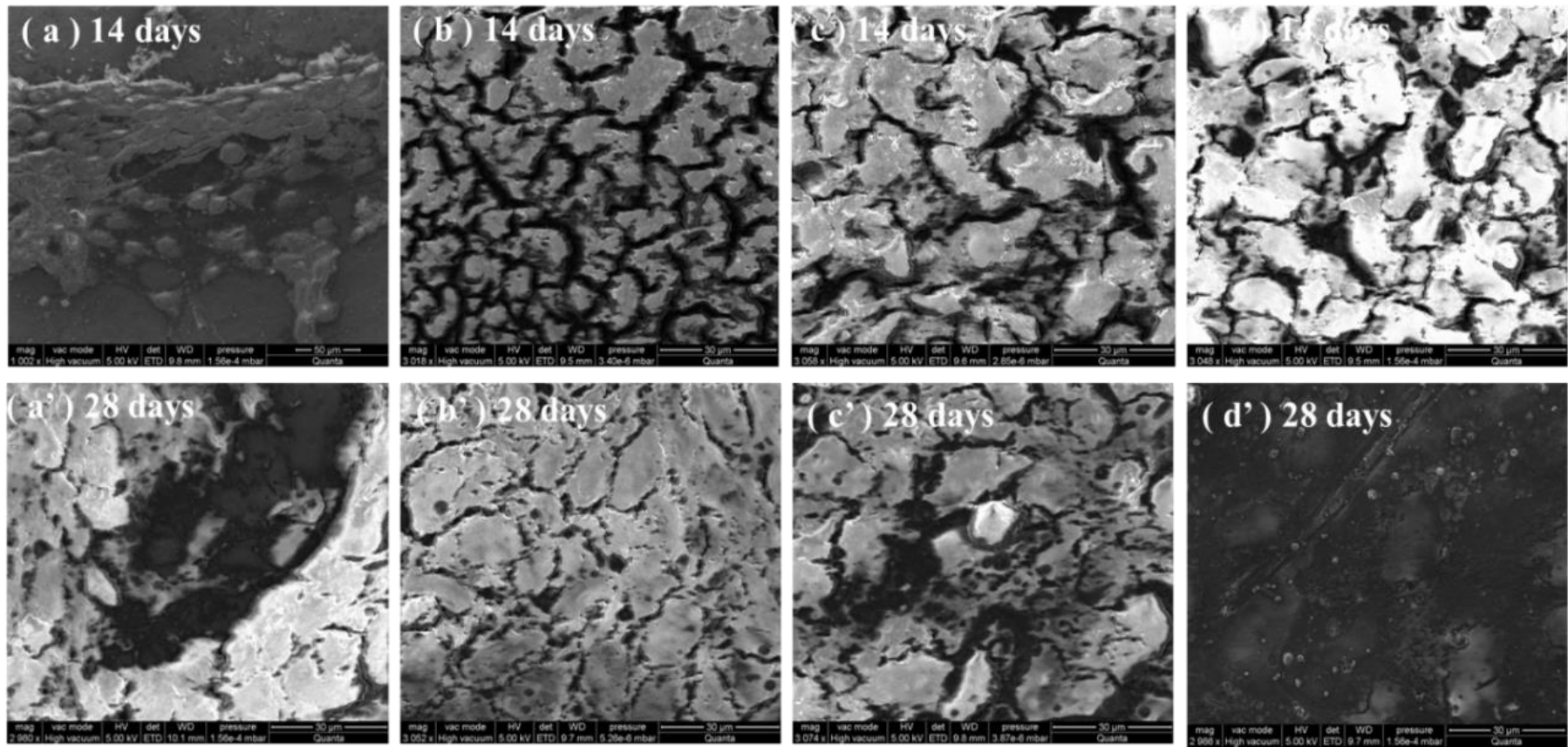


Figure 3.26 : Mineralized structures on the (a,a') TiN , (b,b') 8.1 at % Mg²⁺ , (c,c') 11.31 at % Mg²⁺ and (d,d') 28.49 at % Mg²⁺ containing (Ti,Mg)N thin film coated surfaces.

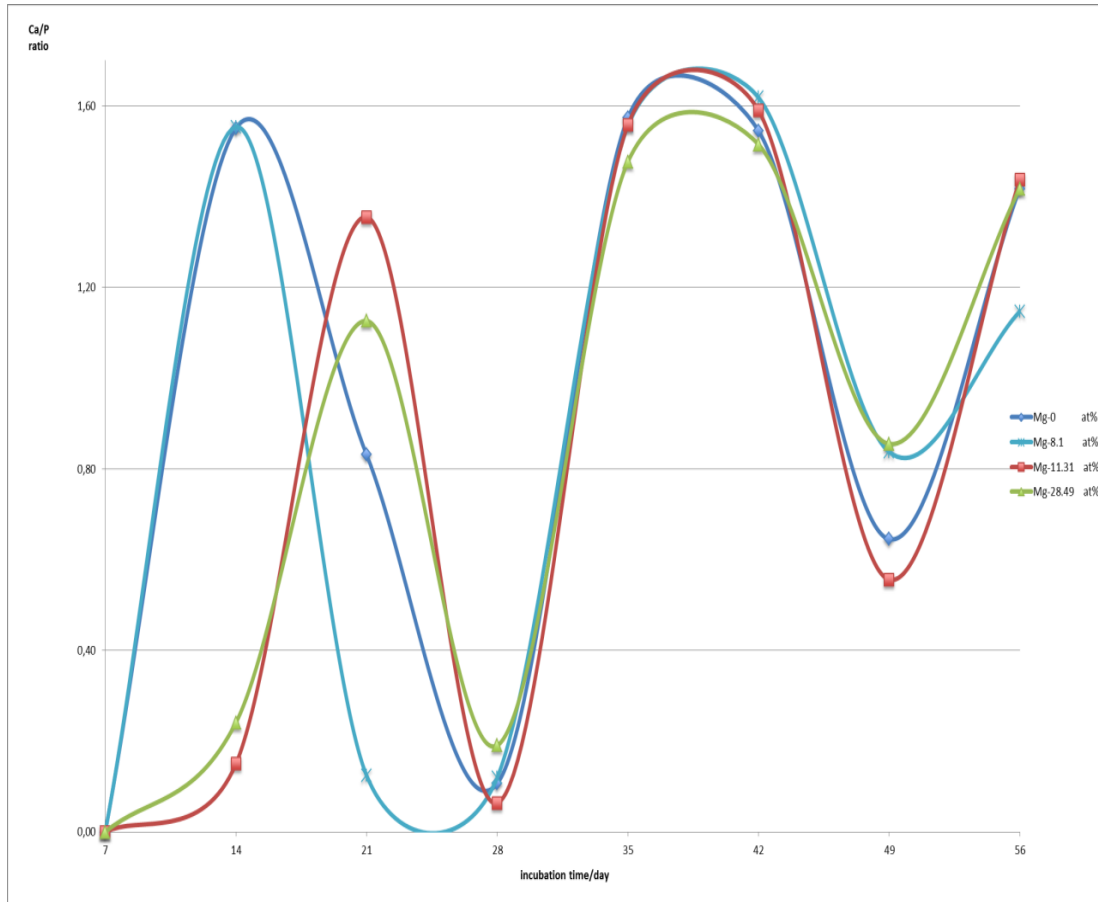


Figure 3.27 : Ca/P ratios on the cell containing TiN and (Ti,Mg)N thin film coated surfaces.

A similar cycle on Ca/P ratios was obtained in HA deposition studies made with the cell free surfaces. Detachment of HA deposits from the cell free surfaces after a critical thickness or dissolution because of the pH change might result in a change in Ca/P ratios and led fluctuations on Ca/P ratio. Furthermore, cell culture environment does not entirely simulate the dynamics of *in vivo* conditions, so long term cell culture studies on surfaces might not be able to give a conclusive result after 21-28 days.

3.8.3. XRD analysis

More crystalline HA structures were obtained when the cells were introduced to the cell culture medium. In the absence of cells, the only TiN peak obtained was around 36.27 °. A new high intensity HA peak, which was not observed in the absence of the cells, was obtained at around 31.67 ° (Figure 3.28). Therefore, it was clear that, cell presence affected the HA deposition process on the thin film coated Ti substrates in

a positive manner and more crystalline hydroxyapatite structures were obtained on surfaces.

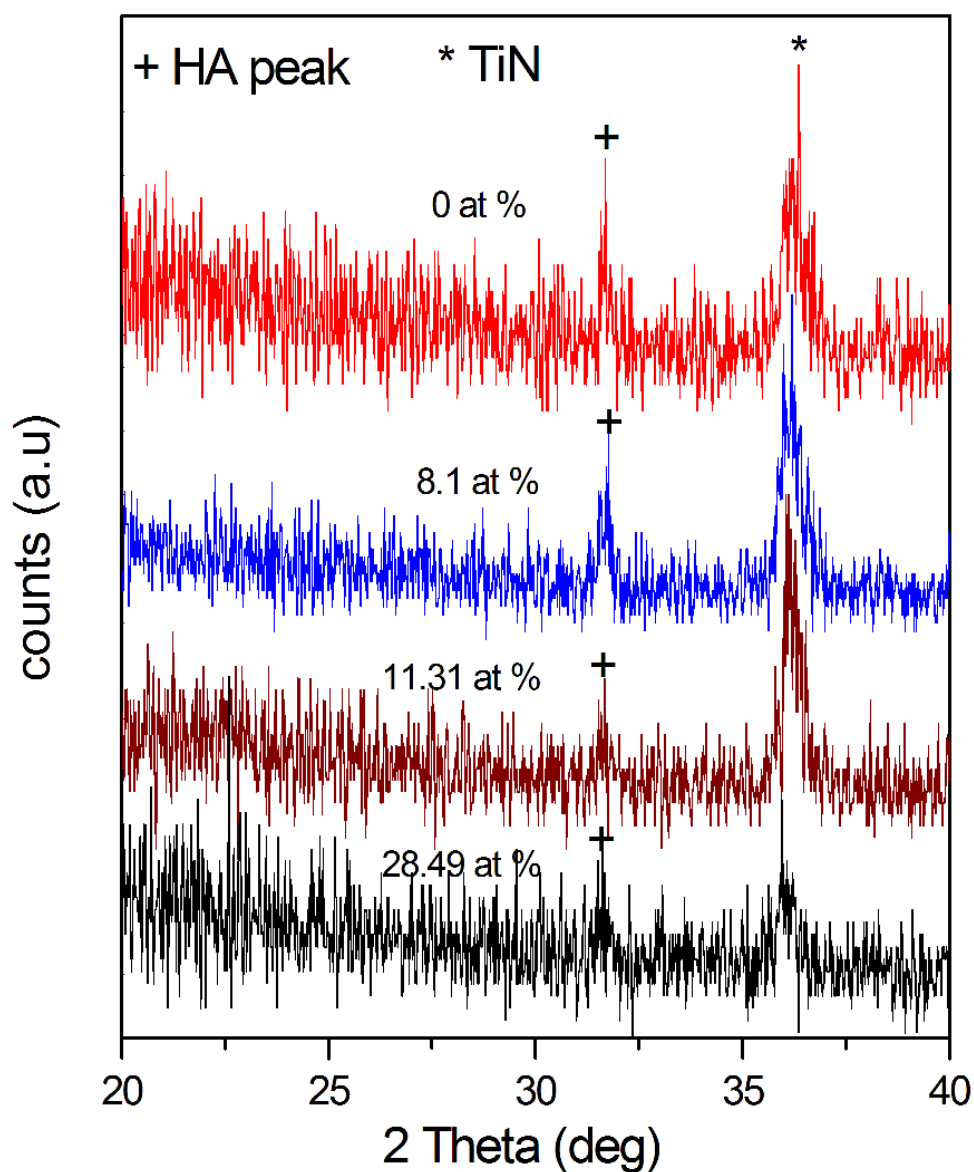


Figure 3.28 : XRD diffraction pattern of (Ti,Mg)N and TiN thin film coated surfaces in the presence of cells (35 days).

3.8.4. FTIR analysis

Roessler, et al., (2001) made a detailed FTIR analysis of collagen matrix, mineralized collagen matrix, HA and bone in order to determine the specific FTIR profile (Figure 3.29).

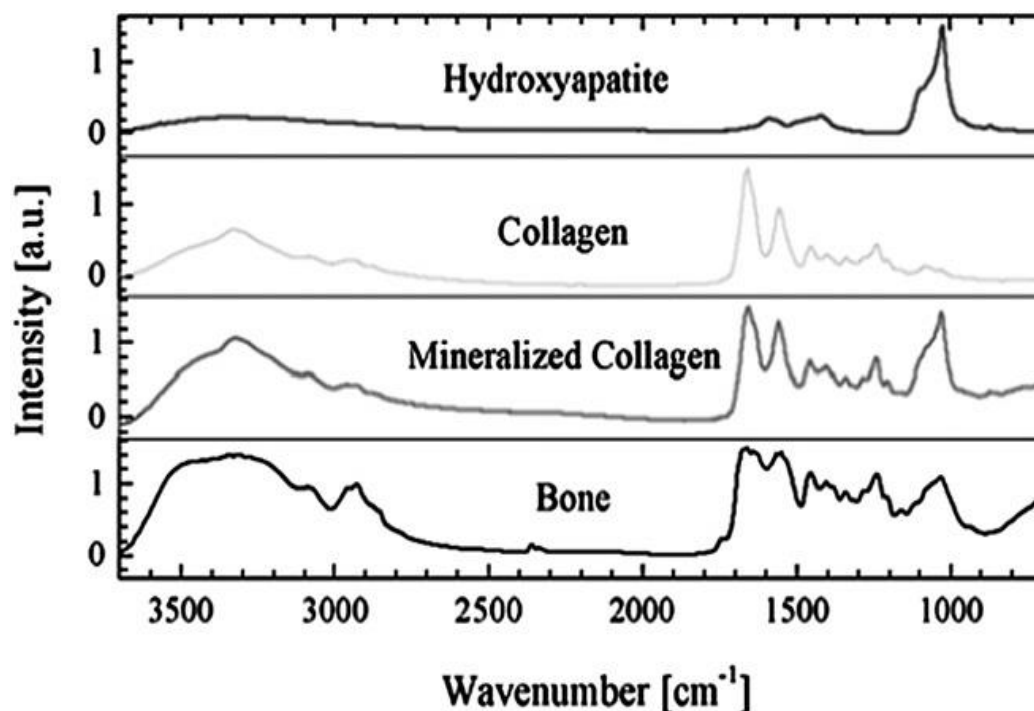


Figure 3.29 : FTIR spectrum of a mineralized collagen in comparison to spectra of pure HA, collagen, and native bone (Roessler et al., 2001)

In the collagen matrix, peaks around 1660, 1555 and 1280 cm^{-1} belong to the amide I, amide II and amide III groups respectively (Roessler et al., 2001). When the collagen is mineralized, HA peaks also started to appear, especially PO_4^{3-} peaks around 1090 cm^{-1} .

In this study, FTIR analysis was done for each sample that was incubated with cells for 7-56 days (1-8 weeks). In cell culture medium, when cells were not present, HA deposition was shown to be started during the first week (Figure 3.24). When the cells were introduced to the medium, not only cell culture medium but also cells would be responsible for the HA deposition. Collagen peaks around 1545 and 1250 cm^{-1} (amide II and amide III, respectively) were obtained from extracellular matrix that were secreted because of osteoblast activity on all surfaces in 7 days (Figure 3.30a).

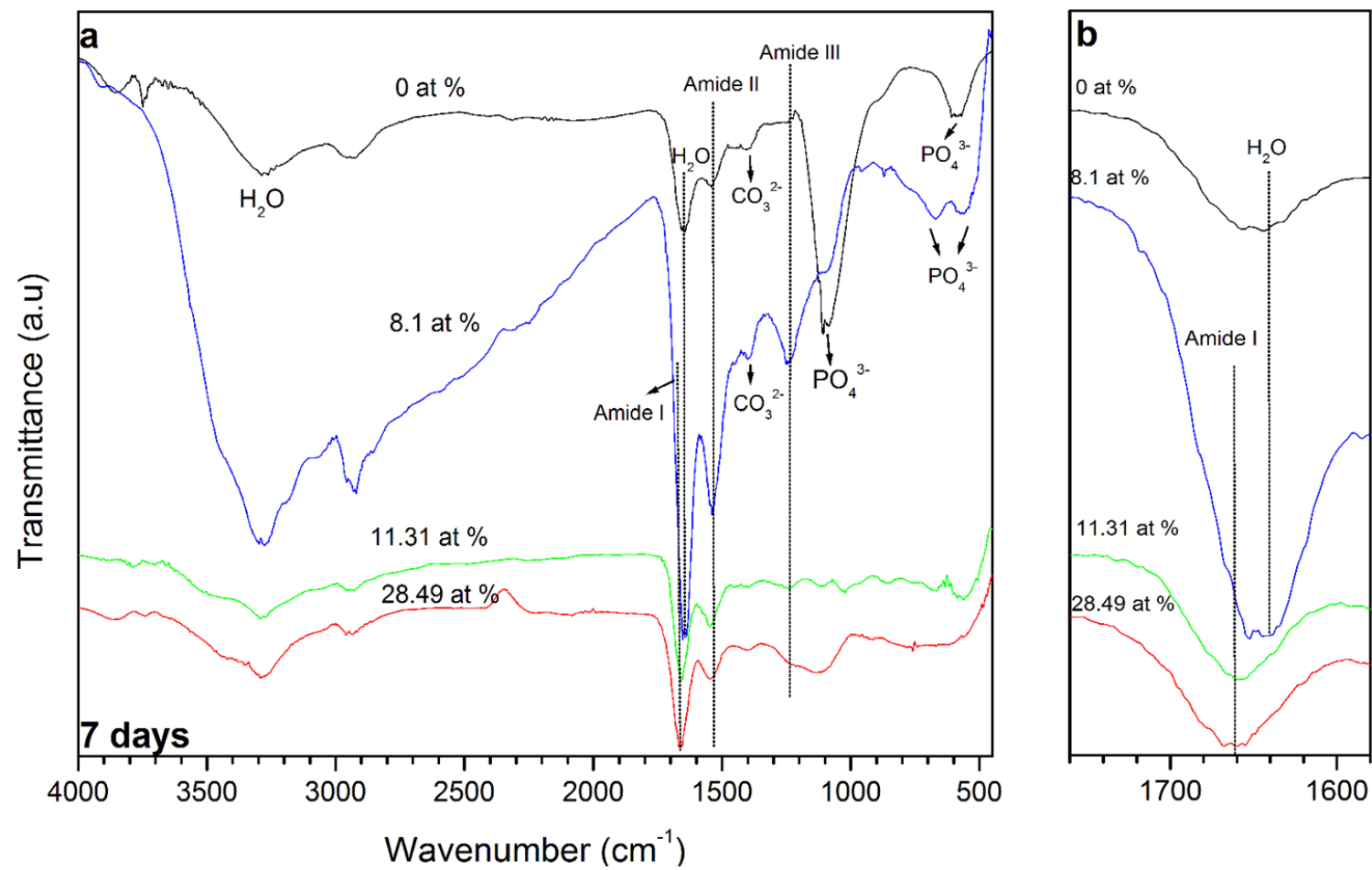


Figure 3.30 : FTIR spectrum of a mineralized collagen on TiN and various Mg^{2+} doped (Ti,Mg)N surfaces after 7 Days.

Besides the collagen peaks, characteristic HA peaks such as PO_4^{3-} , CO_3^{2-} and H_2O were also obtained on TiN (0 at % Mg^{2+}) and low Mg^{2+} (8.1 at %) doped (Ti,Mg)N thin film coated surfaces. This finding indicated that mineralization started on these samples in 7 days. On the other hand, no HA peak but collagen peaks (amide I, amide II and amide III) were detected on 11.31 and 28.49 at % Mg^{2+} doped TiN surfaces. It is clear that when collagen matrix was mineralized, amide I groups disappeared (Figure 3.30b). Therefore, it could be said that high amount of Mg^{2+} presence (11.31 and 28.49 at %) made a negative effect on mineralization process.

In SEM micrographs, it was showed that there was a thick mineralized structure on all samples after 35 days (Figure D.5). Collagen peaks around 1545 and 1248 cm^{-1} (amide II and amide III) were obtained on these samples in FTIR analysis (Figure 3.31a). Furthermore, PO_4^{3-} peak that is an indicator of HA deposition and mineralization, was detected around 1074 cm^{-1} on all samples after 35 days. Another characteristic HA peak, CO_3^{2-} at around 1460 cm^{-1} , was also obtained on all samples in FTIR analysis. CO_3^{2-} peaks substitute for phosphate ions (Figure 3.31a) in HA structure (Meejoo, et al.,2006). When the phosphate amount and crystallinity of HA structure increased, PO_4^{3-} peaks became more obvious. Although PO_4^{3-} peak was only obtained around 1074 cm^{-1} after 35 days, PO_4^{3-} peaks around 579, 608 and 1116 cm^{-1} were obtained after 42 days (Figure 3.31b). NO_3^- peak that is known as indicator of residue synthesis for HA deposition (Raynaud et al., 2002) was also obtained around 1397 cm^{-1} after 35 days. This peak disappeared when calcification increased during 42 days (Figure 3.31b). Pyrophosphate groups, $P_2O_7^{4-}$, was also obtained around 720 cm^{-1} on all samples in FTIR analysis (Figure 3.31a). Nevertheless, these peaks disappeared after 42 days (Figure 3.31b). In literature, $P_2O_7^{4-}$ groups were disappeared when the samples were exposed to heat treatment to increase crystallinity (Raynaud et al., 2002). It could therefore be concluded that the matrix on the surfaces did not contain completely crystalized HA structures after 35 days and crystalline HA could only be obtained after 42 days of incubation. HPO_4^{2-} peaks also obtained around 877 cm^{-1} after 42 days (Figure 3.31b). This peak is also a characteristic HA peak and give information about the HA crystal structure and indicated that there was still Ca^{2+} deficiency in HA structure (Kwon et al.,2003).

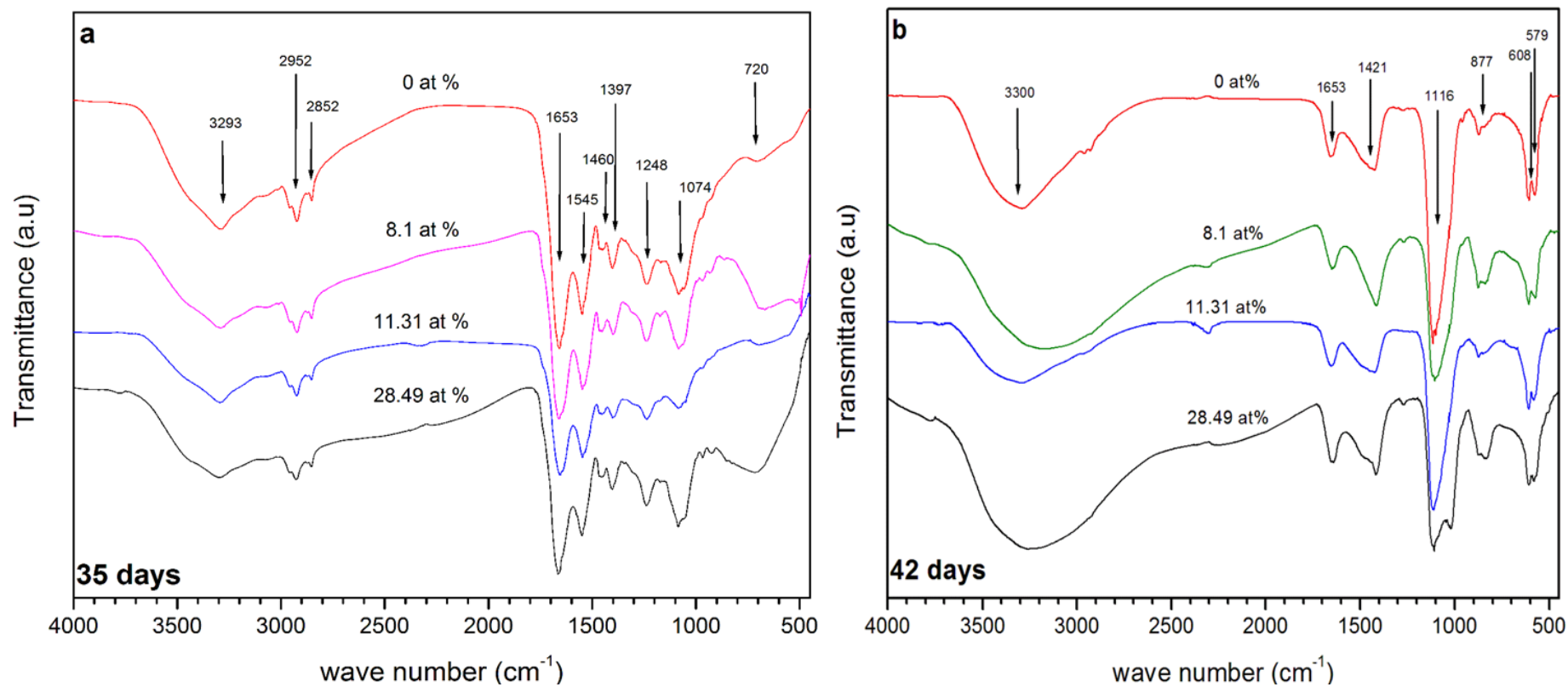


Figure 3.31 : FTIR analysis made on cell containing TiN and (Ti,Mg)N thin film coated surfaces after a) 35 days and b) 42 days.

This deficiency increased with the increasing Mg^{2+} amount in thin film coatings (Figure 3.31b). $-\text{OH}$ peaks and absorbed water peaks are another characteristic HA peaks. $-\text{OH}$ peaks around 1653 cm^{-1} were obtained after 35 and 42 days in FTIR analysis. It was showed that when the collagen was mineralized, amide I peak dissapperaed and H_2O peak observed (Figure 3.30b) in 7 days. Similar results were obtained after 35 days (Figure 3.32). Amide I peak was only observed on high Mg^{2+} (28.49 at %) containing surfaces on which Mg^{2+} lead to an slowdown in cell proliferation. Moreover, not only amide I group but also amide II and amide III peaks dissapperared when crystalline HA structures deposited on surfaces (Figure 3.31b).

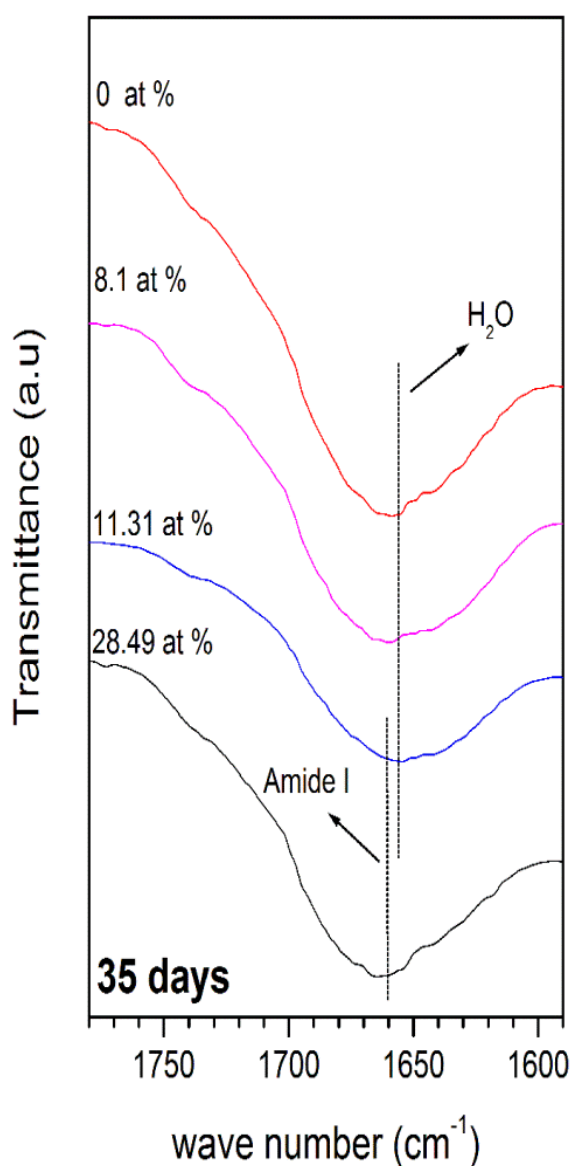


Figure 3.32 : Collagen peak (amide I) dissappeared when HA crystallinity increased.

4.CONCLUSIONS

In this thesis, the aim was to produce Ti hard tissue implants with better osteointegration and corrosion properties and to better understand the effect of Mg and surrounding cells in the HA deposition on implant surface. For this, TiN and (Ti,Mg)N thin film coatings were deposited on Ti substrates by using cathodic arc PVD technique. The XRD analysis of the $(\text{Ti}_{1-x},\text{Mg}_x)\text{N}$ coatings indicated that addition of Mg^{2+} equivalent to $x = 0.064$ did not form a separate Mg or magnesium nitride phases. However, Mg^{2+} doping into TiN thin film coatings resulted in a shift in TiN peaks to lower diffraction angles.

HA deposition studies were conducted in two parts. In the first part, magnesium substituted hydroxyapatite (Mg-HA) was synthesized in different simulated body fluid concentrations (1X, and 5X), and characterization of samples was done by SEM, EDS and FTIR analysis. The results showed that nucleation for HA synthesis started in the first week on (Ti,Mg)N surfaces on 1XSBF medium, but homogenous HA structure could not occur in both (Ti,Mg)N and TiN surfaces even after 4 weeks. Nevertheless, local HA deposition on Mg^{2+} doped TiN surfaces were higher than TiN surfaces. Besides this, FTIR analysis showed that Mg^{2+} presence in thin film coatings accelerated HA deposition in consequence of Mg^{2+} dissolution into the simulated body fluid during the HA formation process. In other words, the Mg^{2+} doped TiN structure acted as a magnesium source promoting the Mg-substituted HA formation in simulated body fluids. When the Mg^{2+} released from the thin film coatings, diffraction angle that belong to TiN peak of (Ti,Mg)N coatings turned back to its original position after the first week. This type of uneven HA deposition would make the cell proliferation and characterization difficult since it is not reproducible. To solve this problem, more concentrated SBF (5X) were used for HA deposition studies. Homogenous HA coatings on both (Ti,Mg)N and TiN surfaces were obtained in 3 days after 5XSBF incubation. FTIR analysis showed that Mg^{2+} substitution into HA structure resulted in lower intensity for PO_4^{3-} groups and higher intensity for CO_3^{2-} groups. These findings are in agreement with the literature

and accepted as clues for Mg^{2+} doping into HA structure since Mg^{2+} doping into HA lattice lead to a decrease in crystallinity and lowers the intensity of PO_4^{3-} peak. Moreover, Mg-HA granule size was found to be larger (i.e. 2-3 times) than HA granule size. A shift in TiN peaks were also observed at XRD analysis in (Ti,Mg)N coatings after Mg-HA deposition like in the case of 1X SBF. For a better understanding, Mg-free 5XSBF studies were conducted. Since, there was no HA deposition on the TiN surfaces in Mg-free SBF, while HA deposition occurred on Mg^{2+} doped TiN surfaces in Mg-free SBF, it was concluded that Mg^{2+} in the coatings acted as Mg^{+2} source for HA deposition. It is known that Mg^{+2} is necessary for HA deposition on implant surfaces and these results clearly showed the necessity of Mg^{2+} presence.

Although chemical and topografical characterization is important, cell attachment and proliferation on different surfaces should also be studied to relate the changes in the surface to the biological performance. Cell proliferation assay (MTS) results showed that cell proliferation was higher on Mg-HA deposits that were synthesized on low Mg^{2+} (4.24 at %) containing (Ti,Mg)N surfaces compared with the HA deposits that were synthesized on TiN surfaces. However, Mg-HA deposits that were synthesized on high Mg^{2+} (10.42 at %) containing surfaces had a negative effect on cell proliferation. Therefore, Mg^{2+} in the thin film coatings should be below 10 at % for better biological performance. Accelerated corrosion rate of thin film coatings with Mg^{2+} amount higher than 10 at % could be the other reason for the decrease in cell proliferation.

In the second part of the study, in order to better mimic the physiological environment, cell culture medium was used in HA deposition studies instead of SBF both in the presence and absence of cells. Effect of Mg^{+2} on the cell proliferation and corrosion were also determined in this part. Studies made to determine the effect of cell presence on HA deposition on TiN and (Ti,Mg)N coatings in cell culture medium showed two important results.

Firstly, Mg^{2+} doping into TiN surfaces, especially in high amount (11.31 and 28.49 at %), resulted in a delay of the HA deposition and accelerated corrosion. Furthermore, Mg^{2+} presence on surfaces resulted in protein absorption on to the surfaces. Mg^{2+} free TiN coatings and low Mg^{2+} (8.1 at %) doped TiN surfaces seemed to be better to obtain early HA deposition. However, crystallinity of HA structures were low. Besides this, it was shown that cell presence make a positive effect on the crsytalline

HA deposition and cell proliferation was better on low Mg^{2+} (8.1 at %) surfaces. This makes low Mg^{2+} containing (Ti,Mg)N coatings more attractive than bare TiN coatings for hard tissue implant applications.

Secondly, in the first part, HA deposition studies were done in different SBF concentrations and 1X SBF has almost same chemical ingredients with similar percentages compared to cell culture medium. Cell culture medium additionally contains amino acids, proteins etc.. When samples were incubated in SBF (36.5 °C, pH: 7), homogeneously distributed HA coating could not be obtained even in 4 weeks on TiN surfaces, while HA deposits were successfully obtained on TiN surfaces after the first week in cell culture medium. Incubation conditions of samples (CO_2 level of 5 % and 37 °C) and the other ingredients in cell culture medium might be the reason for the change in HA deposition process. Another finding was that high Mg^{+2} (11.31 and 28.49 at %) doped TiN surfaces showed high corrosion rates and this may also contribute to *in vivo* performance of the implant.

In sum, Mg^{2+} doped thin film coated Ti implants were produced with better biological performance. These new coatings seem to promote HA deposition, cell proliferation and extracellular matrix formation on the surfaces. These encouraging results offer the potential to produce hard tissue implants with the ability to induce on-site HA production on the surface, allowing improved tissue integration.

4.1. Future Prospects

Although the effect of Mg presence on (Ti,Mg)N thin film coatings on cell proliferation and HA deposition was shown, the mechanisms at molecular level are still unknown. Therefore, a detailed study at the molecular level should be done in order to better understand which genes are repressed or activated with the Mg^{2+} presence. Furthermore, in this thesis, osteoblast cells, i.e. differentiated cells, were used so studies could only be focused on cell proliferation, but not to cell differentiation. Studies have now being carried out with stem cells to evaluate the performance of the (Ti,Mg)N coatings on cell differentiation. These studies will give a whole picture on the mechanism of HA deposition on (Ti,Mg)N coatings.

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APPENDICES

APPENDIX A: Plate, Cell Line, Cathodes and Chemicals

APPENDIX B: Solution and Buffers

APPENDIX C: Laboratory Equipment

APPENDIX D: Supplementary Graphs

APPENDIX E: Supplementary Tables

APPENDIX A: Plate, Cell Line, Cathodes and Chemicals

Name	Suppliers
Titanium Plate (Grade II)	
Cell Line	
osteoblast	
Cathodes	
Titanium	Öncel Yüzey Teknolojileri
Magnesium	Öncel Yüzey Teknolojileri
Chemicals	
NaCl	Sigma-Aldrich
NaHCO ₃	Sigma-Aldrich
KCl	Sigma-Aldrich
K ₂ HPO ₄ .3H ₂ O	Sigma-Aldrich
MgCl ₂ .6H ₂ O	Sigma-Aldrich
HCl	Sigma-Aldrich
CaCl ₂	Sigma-Aldrich
Na ₂ SO ₄	Sigma-Aldrich
(CH ₂ OH) ₃ CNH ₂	Sigma-Aldrich
Primocin	Invivogen
DMEM (1 g/L glucose, no phenol red)	Gibco – Invitrogen
MTS (96 [®] AQueous Non-Radioactive Cell Proliferation Assay)	Promega
Phalloidin Alexa Fluor® 546	
FBS	Gibco – Invitrogen
Cacodylic Acid Sodium Salt Trihydrate	Appli Chem
Glutaraldehyde	Sigma-Aldrich

APPENDIX B : Solution and Buffers

- **Cell Culture Medium Preparation**

Cell culture medium (500 ml) were prepared as follows: 50 mL of the medium (DMEM, 500 ml) was removed from the bottle, and 50 mL of FBS and 500 μ L of primocin were added to the bottle.

- **Cacodylate Buffer**

Cacodylate buffer was prepared as follows: 16 g of Cacodylic Acid Sodium Salt Trihydrate was dissolved in 1 L of distilled water, and then pH was adjusted to 7.4.

- **Glutardethde Solution**

Glutaraldehyde solution was prepared from the stock solution of 25% by diluting it to 2.5% with cacodylate buffer.

APPENDIX C : Laboratory Equipment

PVD :	Öncel Yüzey Teknolojileri
SEM with EDS:	JEOL JSM 7000F
XRD:	Philips Model PW3710
FTIR:	Perkin Elmer
Fluorescence Microscopy:	Nikon Eclipse TE 200
CO₂ Incubator:	Thermo Scientific, Steri-Cycle CO ₂ Incubator
Centrifuge:	Beckman Coulter, Microfuge 18
Optical Profilometer:	
Elisa Plate Reader:	Bio-Tek, Elx800
Balances:	Precisa 620C SCS Precisa 125 A SCS
Refrigerators:	-80°C Heto Ultrafreeze 4410 -20°C Arçelik 209lt +4°C Arçelik
Pipettes:	Gilson pipetteman 10 µL, 20 µL, 200 µL, 1000 µL Volumate Mettler Toledo 10 µL, 20 µL, 200 µL, 1000 µL Eppendorf research 2,5 µL 10 µL, 20 µL, 200 µL, 1000 µL
pH meter:	Mettler Toledo MP220
Spectrophotometer:	PerkinElmer Lambda25 UV/VIS Spectrometer
Waterbaths:	Memmert wb-22

APPENDIX D : Supplementary Graphs

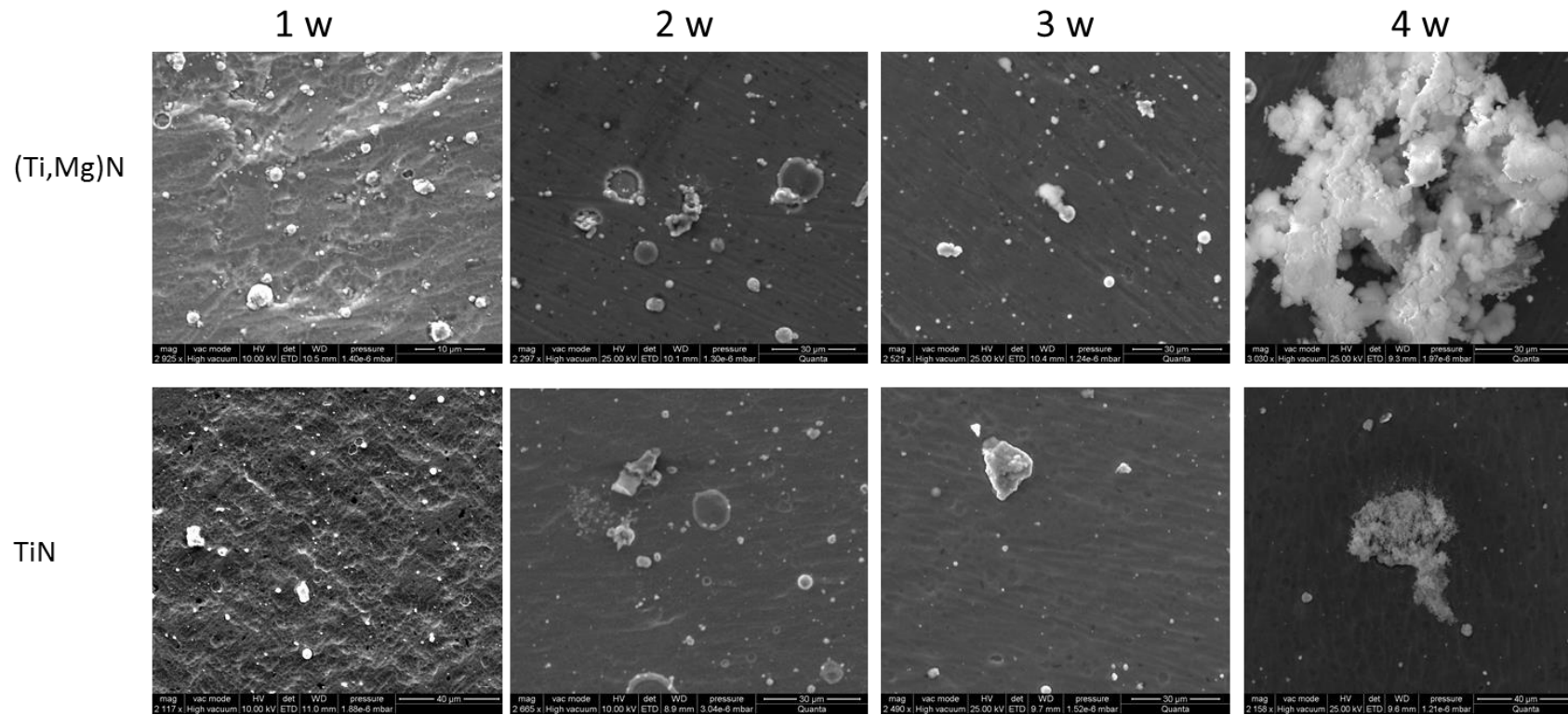
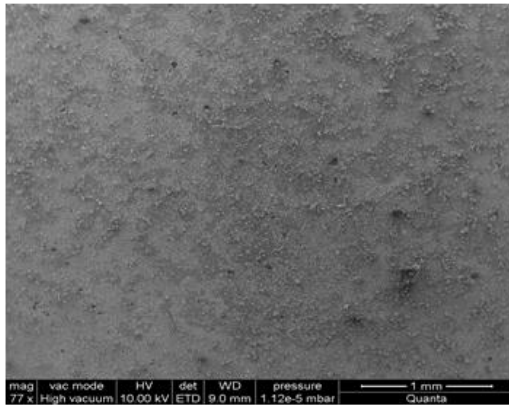


Figure D.1 : SEM micrographs of (TiN) and (Ti,Mg)N surfaces after 1X SBF incubation.

(Ti,Mg)N



TiN

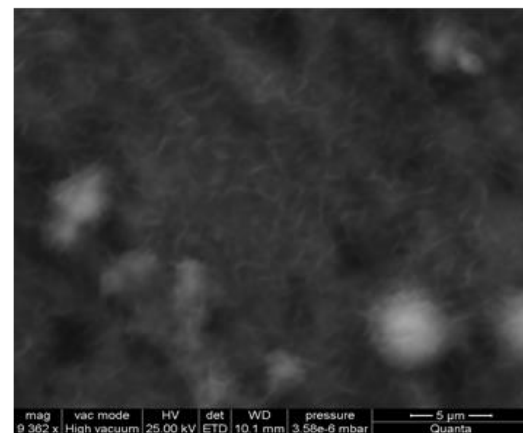
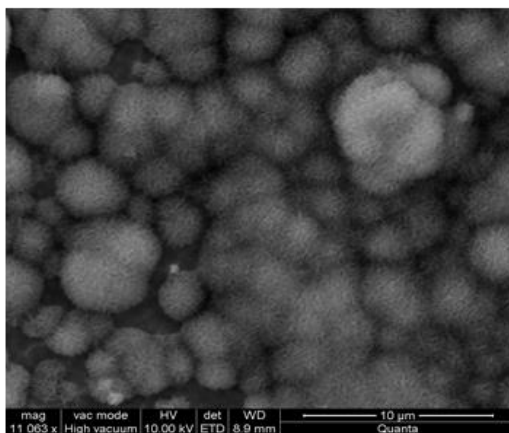
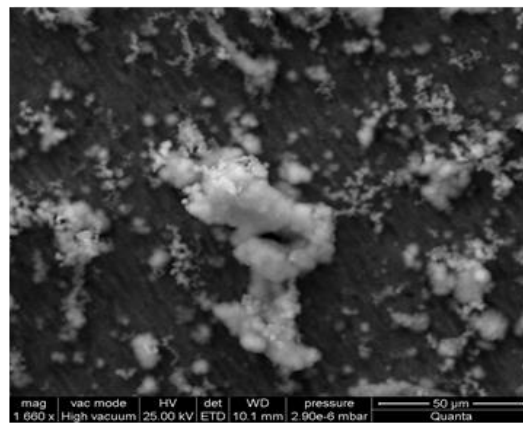
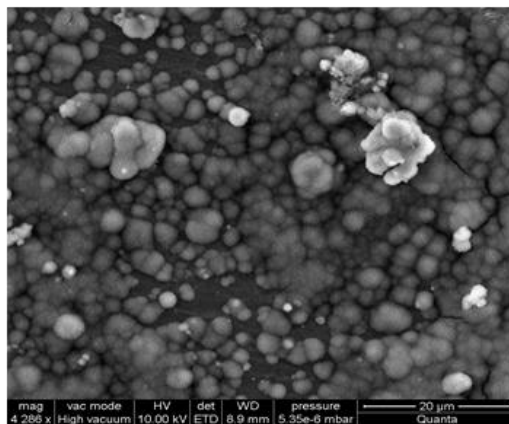
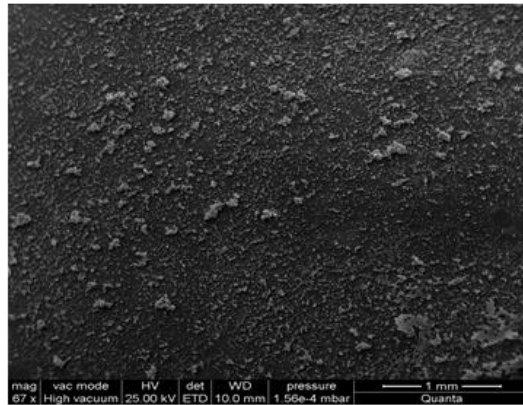


Figure D.2 : SEM micrographs of thin film coatings after 5X SBF incubation.

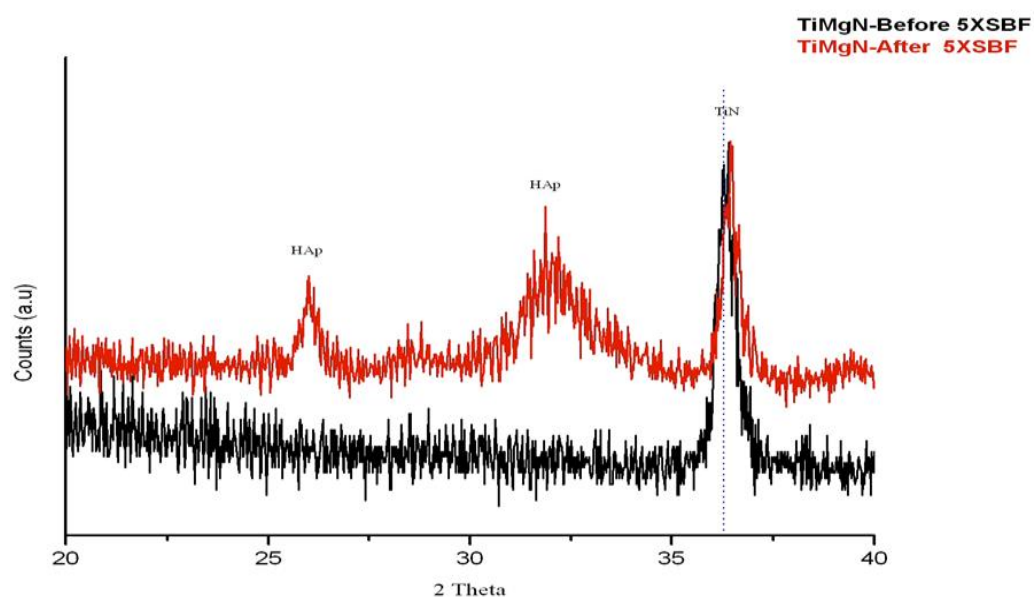


Figure D.3 : Shift in TiN peak on (Ti,Mg)N surfaces after 5X SBF incubation.

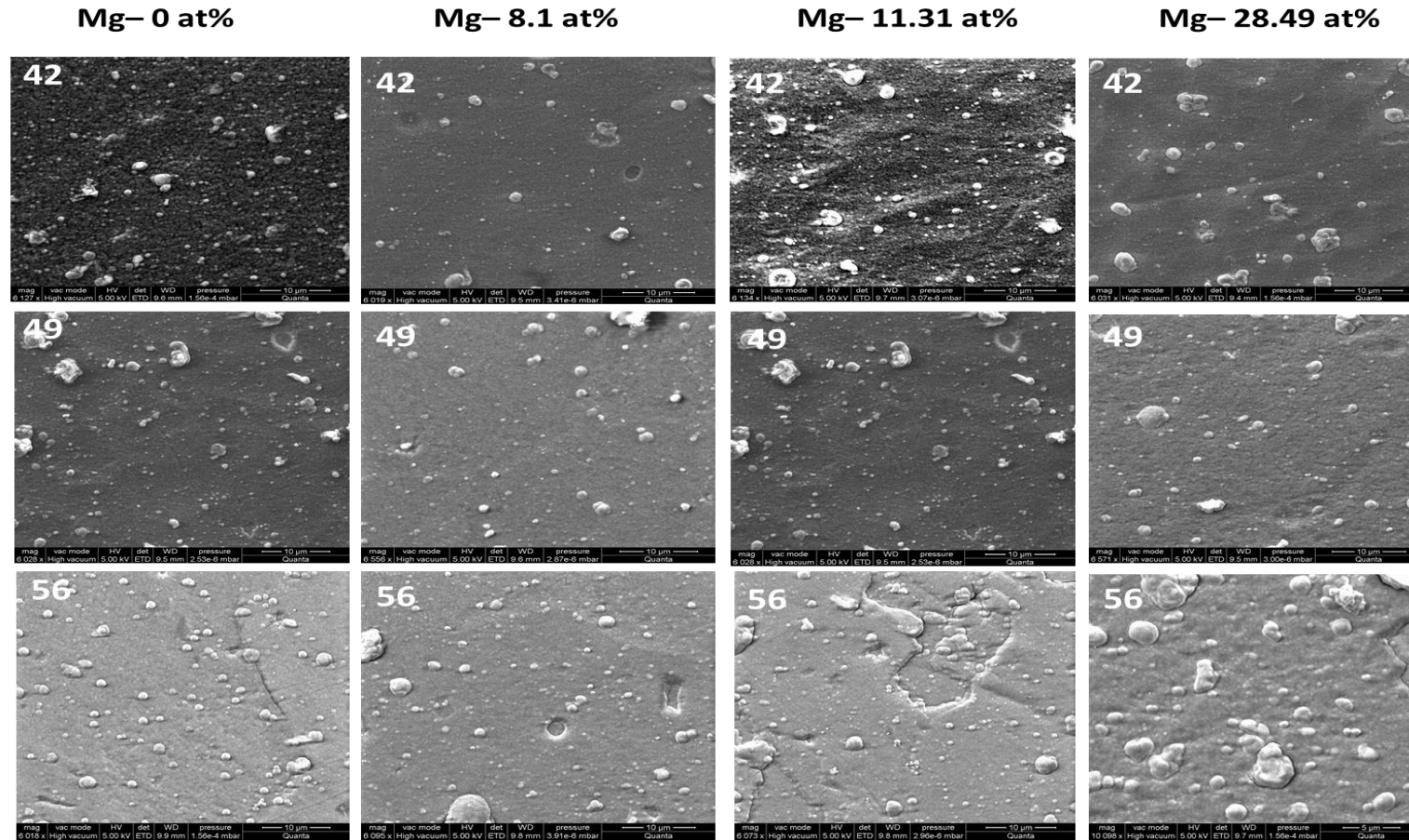


Figure D.4 : Supplementary SEM micrographs of cell free (TiN) and (Ti,Mg)N surfaces with different Mg²⁺

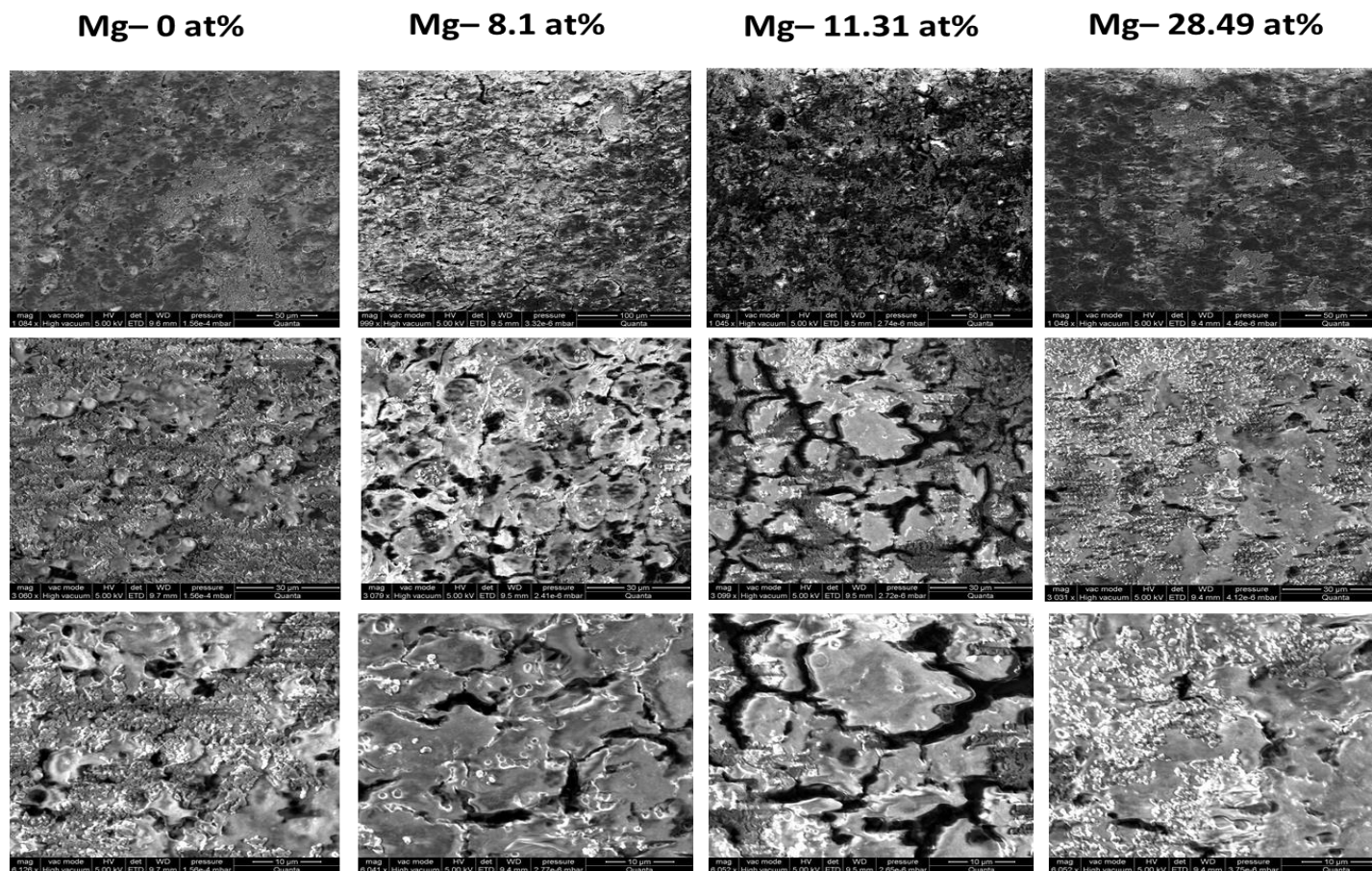


Figure D.5 : Supplementary SEM micrographs of cell containing (TiN) and (Ti,Mg)N surfaces with different Mg²⁺ contents after 35 days.

APPENDIX E : Supplementary Tables

Table E.1 : Elemental analysis of (Ti,Mg)N coatings after 5X SBF incubation.

	In HA Structure		In Background	
	% Atomic	% Weight	% Atomic	% Weight
Mg	4.38	2.64	10.01	5.60
Ca	38.65	38.39	11.07	10.22
P	20.66	15.86	7.43	5.30
Ti	36.32	43.11	71.50	78.88

Table E.2 : Elemental analysis of TiN coatings after 5X SBF incubation.

	In HAp Structure		In Background	
	% Atomic	% Weight	% Atomic	% Weight
Mg	-	-	-	-
Ca	62.33	66.60	10.73	9.38
P	32.56	26.88	7.12	4.81
Ti	5.11	6.52	82.15	85.81

Table E.3 : Elemental composition of TiN and various Mg²⁺ doped TiN surfaces in the presence and absence of cells

Cell Free Surfaces							Cell Containing Surfaces				
Day	Ca	P	Mg	Ti	Ca/P	Mg (at %)	Ca	P	Mg	Ti	Ca/P
7	1,95	1,22	0,40	96,43	1,60	0	0,00	0,00	0,00	100,00	0,00
14	5,50	3,46	0,32	90,73	1,59		4,96	3,20	9,71	82,13	1,55
21	13,78	9,42	0,86	75,94	1,46		0,33	0,39	3,37	95,91	0,83
28	22,22	15,30	1,21	61,26	1,45		1,83	17,14	50,28	30,74	0,11
35	6,23	56,12	5,46	32,19	0,11		27,35	17,39	1,35	53,91	1,57
42	30,98	19,75	6,76	42,51	1,57		28,02	18,14	2,20	51,65	1,54
49	37,31	24,15	2,46	36,09	1,55		0,24	0,37	0,23	99,17	0,64
56	45,34	29,74	2,92	22,01	1,52		9,65	6,81	34,55	48,98	1,42
7	0,39	0,33	5,02	94,26	1,19	8,11	0,00	0,00	4,90	95,10	0,00
14	0,00	0,26	5,49	94,25	0,00		6,15	4,13	7,12	82,60	1,49
21	9,76	6,49	6,55	77,20	1,50		0,34	2,90	20,90	75,86	0,12
28	21,12	13,85	4,83	60,20	1,53		0,12	0,22	8,05	91,61	0,56
35	1,47	24,64	5,74	68,16	0,06		25,49	16,15	4,96	53,41	1,58
42	25,60	17,02	10,27	47,12	1,50		29,10	19,57	4,76	46,57	1,49
49	40,02	25,28	5,10	29,60	1,58		0,33	0,39	6,47	92,19	0,83
56	41,52	25,54	4,23	27,72	1,63		0,60	0,48	4,93	93,99	1,26
7	0,00	0,00	12,88	87,12	0,00	11,42	0,00	0,00	15,04	84,96	0,00
14	0,00	0,00	19,03	80,98	0,00		0,00	0,00	14,64	85,36	0,00
21	0,44	0,30	14,59	84,68	1,47		0,66	0,49	14,37	84,48	1,35
28	11,41	7,39	10,51	70,68	1,54		0,60	9,40	30,72	59,28	0,06
35	0,28	1,39	10,40	87,93	0,20		15,93	10,23	9,44	64,41	1,56
42	8,45	4,89	9,90	76,77	1,73		30,48	19,18	4,50	45,84	1,59
49	29,15	19,30	9,89	41,66	1,51		0,14	0,26	16,65	82,95	0,56
56	45,59	29,53	5,13	19,75	1,54		3,35	2,33	16,29	78,03	1,44
7	0,00	0,00	35,05	64,95	0,00	28,49	0,00	0,00	35,05	64,95	0,00
14	0,00	0,00	33,53	66,47	0,00		0,66	2,72	57,86	38,76	0,24
21	0,15	0,06	36,66	63,13	2,48		1,38	1,22	36,25	61,15	1,13
28	6,39	4,09	30,43	59,09	1,56		0,81	4,25	48,04	46,90	0,19
35	3,40	58,66	9,74	28,20	0,06		21,19	14,36	20,30	44,15	1,48
42	33,65	21,81	30,05	14,50	1,54		23,23	15,34	21,57	39,87	1,51
49	33,38	21,23	19,57	25,81	1,57		0,34	0,40	30,89	68,37	0,86
56	58,79	23,98	14,03	23,20	2,45		9,65	6,81	34,55	48,98	1,42

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- Researcher, Detection of Citrate using Electrochemical Impedance Spectroscopy by different Circuit Modeling (2011-2012)
- Researcher, Design, Fabrication and Characterization of Biosensor through Integration of Micro Channel System on Non-woven Textile Surfaces (2012-2014)
- Istanbul Technical University Travel Award (2012,2013)

PUBLICATIONS/PRESENTATIONS ON THE THESIS

- **S.Onder**, A.C. Calıkoglu, K. Kazmanli, M. Urgan, G.T.Kose, F.N.Kok, Effect of Osteoblast Presence on Hydroxyapatite Formation on TiN and (Ti,Mg)N Thin Film Coated Titanium Surfaces (in preparation).
- **S.Onder**, F.N.Kok, K. Kazmanli, M. Urgan, Magnesium Substituted Hydroxyapatite Formation on (Ti,Mg)N Coatings Produced by Cathodic Arc PVD Technique, Materials Science and Engineering C: Materials for Biological Applications, 33(7):4337, 2013.
- **S. Onder**, G. Torun Kose, F. Kok, K. Kazmanli, M. Urgan, Characterization of (Ti,Mg)N Thin Film Coatings Produced Via Physical Vapor Deposition, TMS2013, Biological Materials Science Symposium, Texas, USA, March 2013 (*Oral presentation*).
- **S. Onder**, F. N. Kok, Mustafa Urgan, K. Kazmanli, Construction of (Ti,Mg)N Thin Film Coatings via Physical Vapor Deposition Technique and Their Properties , 4th International Symposium Interface Biology of Implants (IBI) 2012, Germany, May 2012.

LIST OF OTHER PUBLICATIONS/PRESENTATIONS

- **S. Onder**, G. Baysal, F.N. Kok, L. Trabzon, H. Kızıl, B. Karaguzel Kayaoglu, and I. Gocek, Fabrication of a Textile-Based Biosensor Platform to Determine Lactate level in Sweat (in preparation).
- **Sakip Onder**, Fatma Nese Kok, Levent Trabzon, Huseyin Kizil, Burcak Karaguzel Kayaoglu, and Ikilem Gocek, Fabrication of a Textile-Based Platform for Rapid Analyte Detection, Paper ID :CN005, Proceeding of ICPSE 2013, Applied Mechanics and Materials Journal (ISSN: 1660-9336) (*Oral presentation*).
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- Evren Tastan, **S. Onder**, K. Kazmanli, F. N. Kok, Construction of a Biosensor for the Detection of Phenolic Compounds, International Conference on Nano materials and Nano systems (nanomat'09), Istanbul, Turkey, 10-13 August, 2009.
- **S. Onder**, K. Kazmanli, F. N. Kok, Immobilization of Hirudin on PTFE Surface to Increase its Blood Compatibility, 15th International Biomedical Science and Technology Symposium, Guzelyurt, TRNC, 16-19 August, 2009.
- Evren Tastan, **S. Onder**, K. Kazmanli, F. N. Kok, Construction of a Laccase Biosensor, International Symposium on Biotechnology: Developments and Trends (BIOTECHMETU2009), Ankara, Turkey, 27-30 September, 2009.
- **S. Onder**, K. Kazmanli, F. N. Kok, Immobilization of Hirudin on PTFE Surface, International Symposium on Biotechnology: Developments and Trends (BIOTECHMETU2009), Ankara, Turkey, 27-30 September, 2009.

