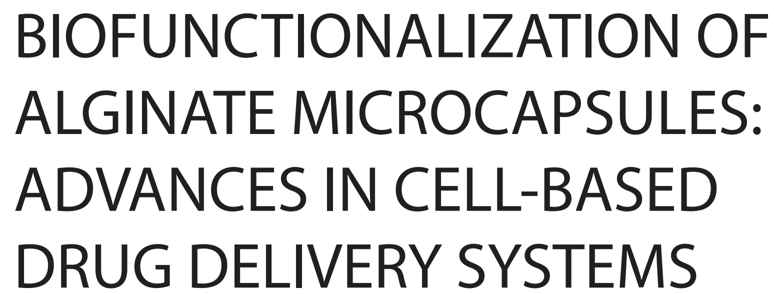
BIOFUNCTIONALIZATION OF ALGINATE MICROCAPSULES: ADVANCES IN CELL-BASED DRUG DELIVERY SYSTEMS

ANE GARATE LETONA

Vitoria-Gasteiz 2015

Universidad del País Vasco
Euskal Herriko Unibertsitatea



BIOFUNCTIONALIZATION OF ALGINATE MICROCAPSULES: ADVANCES IN CELL-BASED DRUG DELIVERY SYSTEMS

ANE GARATE LETONA

Vitoria-Gasteiz 2015

University of The Basque Country (UPV/EHU)
Laboratory of Pharmaceutics
Faculty of Pharmacy

(cc) 2015 ANE GARATE LETONA (cc by 4.0)

Eskerrak
Agradecimientos
Acknowledgements

Tesi hau hastia erabaki nauenetik urte batzuk pasau die, gauza on eta txar guztixekin. Oin, bukaerara helduta, atzera begiratu eta momentu asko gogoratzian bide zail honetan ez nazela inoiz bakarrik sentiru konturatu naz. Beti egon nazela nire inguruko pertsonez babestuta, bai laborategixan eta baitxa laborategitxik kanpo. Horregatik, esker onak emun nahi dotzuet, lan honetan partehartze zuzena euki dozuenoi baina baitxa esperientzia luze honetan nire euskarri izen zarien danoi.

En primer lugar querría agradecer a José Luis Pedraz por darme la oportunidad de trabajar en el grupo de investigación NanoBioCel y seguir en él una vez terminada la tesis. A mis directores de tesis Rosa Hernández y Gorka Orive, agradeceros por haberme guiado en la realización de esta tesis, por la ayuda que me habéis ofrecido sobre todo en los momentos difíciles, por las oportunidades que me habéis brindado y la comprensión que habéis demostrado. Muchas gracias.

Me gustaría también agradecer a todos los integrantes del departamento, a los profesores, Amaia, Jon, Manoli, Arantxa, Alicia, Marian, Ana, Begoña… a los compañeros de tecnalia, Amanda, Leire Plaza, Fer, Zuri, Noe, Ana Castilla, Sergio, Sonia, Arantxa, Aitziber, Leire Berru, Esti Villén, Esti Blanco….Gracias por todas las dudas que me habéis ayudado a resolver y por vuestra paciencia cuando me paso por el depar a robar o escanear. También querría agradecer a Ángela y Andrés su eficiencia en resolver todas nuestras dudas, su paciencia, amabilidad y sobre todo su buen humor. Muchas gracias a todos!

A mis compañeros del CIEA…gracias a Laura, Jesús y Gustavo por vuestra simpatía, por los "egun on-es" y por demostrar que es posible darlo todo en el trabajo sin morir en el intento. Laura, cuento contigo para elegir el vestido! ;) A las nuevas incorporaciones, Janire, Ainhoa y Ilia, gracias por las ganas de trabajar y la ilusión que transmitís. También quisiera agradecer a todos los demás becarios-amigos que habéis compartido la tesis conmigo: Tatiana, Oihane, Mireia, María, Itxaso, Edi, Paola, Edu, Amaia, Susana, Olaia, María…muchas gracias por las confidencias compartidas, las comidas, por las juergas, los montaditos, Donosti…porque no todo va a ser trabajar!

A los microenkapsulatzailes: Argia, inoiz ezingotzut eskertu tesi hontan nigatik in dozun dana. Ezagutzen doten pertzona onenetariko bat zara, zure lagunen laguna, maite dozunengatik dana emuteko prest dauena. Mila gauza bizi izen dou tesi honetan hasi nintzenetik, eta asko pozten naz zu beti nire onduan egon zarelako. Eskerrik asko zure aholku danengatik, nire duda danak erantzutiagatik, planifikazinuengatik…baina batez be nire laguntxo "Pantxika" izetiagatik. Asko gure zaut. Edorta, milesker urte honeitan erakutzi doztazun danagatik. Asko ikasi dot zurekin eta zugandik. Kanpaian pasautako momentu onengatik, gure bazkari eta sobremesa luziengatik eta zelan ez kit kat bokadiluengatik! Jarraitxu zure ilusinua ipitxen itxen dozun gauza danetan. Tania,

eskerrik asko por tu positivismo excepcional, tu paciencia, tu tranquilidad…creo que eres mi antagonista!jaja…por animar a todo el labo a marcarse unas bachatitas, por ser la coordinadora oficial de los eventos, por tus detalles y tu tarta de tronco de chocolate. Vaya descubrimiento! Pello, (en realidad eres un scaffold-ero pero entras en esta sección,jaja), eskerrik asko por tu buen humor, por tu compañerismo, por ser un barriketero, por animar el ambiente con tus 1000 anécdotas y por intentar que trabajemos bien en el laboratorio. Haritz, asko pozten naz gure taldetxora etorri zinelako. Eskerrik asko zure konfiantzagatik, edozein planetarako prest egotiarren, beti entzuteko eta berbetako prest zazelako zure konsejutxuak emuten eta gure baziladatxuak irribarre batekin jasoten dakitzulako. Ganorazko Stark bat zara, duda barik. ;)

Ainhoai…zu zara tesi hau itxia erabakitzian arrazoietako bat. Laborategi honetan sartu nintzenetik beti pentzau izen dot zelako suertia euki nauen kolaborazino bekan zu nire jefatxoa izetiagatik. Danen inbidixia nintzen! Eskerrik asko danagatik, zure lan itxeko modua erakustiagatik, zure alaitasun eta bizipozagatik, zure laguntzagatik, zure detailetxo politxengatik, animuengatik...Benetan pertsonatxo berezixa izen zara nire bizitzan, euki ahal izen doten ereduik onena, eta beti eukikotzut goguan. Azkenian igo nintzen olatura! Asko maite zaut.

A mis dermos, eskerrik asko! Aunque la vida ya nos vaya separando y no estemos todos en el mismo sitio, me siento muy afortunada por haber compartido con vosotros los años de mi tesis. Enara, milesker zure eskuzabaltasun eta alaitasunagatik. Oso bihotz oneko pertsona zara, beti zure onduan dazenena arduratzen. Asko pozten naz ni horrein artian egotiaz. Gora pintxo ta cupcake goxuak! ;) Silvitxuli, muchas gracias por acogerme tan cariñosamente en el grupo. Te lo digo a ti porque todos sabemos que tú eras la jefa!jaja Por todos tus detalles y regalos, por decir las cosas como son y a la cara y sobre todo por intentar mantener la amistad aunque haya km de por medio. Garazitxo, eskerrik asko taldeko psikologa partikularra izatiagatik. Beti entzuteko prest zazelako ta momentu bakoitzian zer esan bidan eta zer entzun nahi dan dakitzulako. Que rule el verdejillo! Aialis, milesker zure alaitasunagatik eta beste aldera noian bakoitzian irribarre batekin jasotziagatik. Zure etxeko siestatxoengatik eta nire galderak pazientzia danakin erantzutiagatik. Gure irakasletxo akonjuntauena zara duda barik! Martis, eskerrik asko zure xamurtasunagatik

ta zure umore puntuagatik, barre asko itxen dot zure okurrentziekin. Zure familiako etxe danak probetan iztiagatik, Madril, Iruña, Elizondo….momentu oso onak bizi izen dou danetan. Aritz!! Milesker por aguantar todos nuestros cotis y marujeos de estos años, por tu amabilidad y por las risas que nos pudimos echar en la boda de Bel. Siempre nos quedará Narnia! Belo, gracias por la pasión que pones en todo lo que haces. Por no dejar espacio a los silencios incómodos (con todo lo que hablas imposible) y por mantener el contacto a pesar de la distancia. Eskerrik asko a todos por los momentos vividos, habéis sido los mejores compañeros que podía tener!

I would like to thank to Eben Alsberg for giving me the opportunity to be a member of his lab during my stay in Case Western Reserve University. Thank you for your kindness and to be a great director while I was working in Cleveland. I also would like to thank to Oju Jeon all his explanations, his patience with me and all I have learned with him during my stay. To all the members of the lab, undergrads, Robyn, David, Neha, Christian, Suzanne, Lauren, Lucy…Postdocs, Truc and Nguyen; Daniel, Henriette, Rod… and especially to my lovely girls: Alex, Phoung, Anna and Julia. Thank you very much for all your help in the lab, but most importantly thank you for being my friends. Zijie. As far as I`m concerned (haha), we enjoyed a lot of moments in those months, you made me laugh a lot. Thank you so much. To other special people that I met in this experience, Jenny, Jessi, Stephan, Punkaj... I'm glad to meet you guys, you all made my experience amazing! By the way, thank you all to make whatsApp account because of me. Hahah…I will never forget the good moments I spent there: Lab picnic, happy hours, dinners, brunches, Lady Gaga`s concert, Arctic Monkeys, shooting, rock climbing, the house in the beach, Fridays in the museum, critical mass, solstice…such a good memories. I love you. We are going to meet soon!yay!!

A los demás amigos que tuve la suerte de conocer durante mi estancia…Jessica, Nuria, Carmen...gracias por guiarnos por Cleveland, por llevarnos a todos los sitios en coche, por los planes, por Toronto, Chicago… y como no, eskerrik asko/Moltes gràcies a mi Gorkilla ta Laiatxo! Me alegro muchísimo por haberos conocido durante mi estancia y de que ahora forméis parte de mi vida. Laia, moltes gràcies por ser una gran compañera de piso, por todas las confidencias, por adentrarme en el mundo del Japo, por la estancia en Barcelona… Gorka, mila esker nire kontu danak entzutiagatik, zure konfiantzagatik,

squash-ian erakustiagatik, squash osteko (eta tarteko) barriketa luziengatik, Clevelandeko juergatxoengatik…Stocholmora bisita pendiente!

Eta zelan ez, nire beste laguntxo danei urte guzti honeitan zuen partetik jasotako indar, laguntza ta berotasuna eskertu nahi dotzuet. Benetan ez dakitzue zelan aprezietan doten zuen danon gertutasuna, ze ingo nauen nik zueik barik! Nire kuadrilako neska politei, ta katarsiszaliei, Maia, Itsaso, Neka, Jaio, Ira, Aro, Gade, Nere, Xabi, Txefo, Monito…eskerrik asko asuntuana larrei ez ulertu arren tesixai buruz indako galderengatik (normalian xaguekin lotuta, klaro), zuen interesagatik, urte guzti honeitan onduan eukitxiagatik, juerga, trago, afai, poteo, barriketa, sobremesa luze danengatik…nire bizitzako parte zare eta asko pozten naz horregatik. Ibo ta Mimi, zuei bukaerarako itxitzuet aurreko danaz gain tesiko maketazino ta portadan be lagundu doztazuelako. Eskerrik asko benetan, gozada bat da nire albuan zuen moduko jendia eukitxia, jota liauta egon arren beti laguntzeko prest dauena. Animo neskatxak, laister arkitektatxo bikainak izengo zare eta! Azken txanpa ta lixto!!! Asko gure zauet!! Marci, Mirentxi, Mirari, Garbi…zueik nirekin hainbeste ez egon arren asko eskertzen dotzuet niregatik erakusten dozuen interesa eta badakitx beti hor egongo zariela urtiak aurrera doiezen arren!

Garitxoi…tesi honen hasieran ezagutu ginen, eta ordutik 1000 abentura bizi izen dou. Asko lagundu doztazu denbora guzti honetan, eskerrik asko danagatik, nire lanai buruzko ika-mikak pazientzia danaz entzutiagatik, zure interesagatik, animuengatik, nigan sinistiagatik…eskerrik asko zure babesagatik. Asko maite zaut.

Nire familixa zoragarrixai…aita ta ama, eskerrik asko beti nigan euki dozuen konfiantzagatik, nire erabaki danak errespetetiagatik, zuen laguntza danagatik, zuen ulergarritasunagaitxik, etxeko giro alai/xelebriagatik…guraso onenetan onenak zare, asko maite zauet. Idoyingis, nire ahizpatxo maitea, bihotz erraldoi bat daukotzu, eskerrik asko beti hor egotiarren, zure zorokeixa ta xelebrekeixengatik, opera kantuengatik, zure laguntzagatik, barriengatik…Mari! eskerrik asko zure preokupazino ta ardura danengatik, corsita lapurtzen iztiagatik (ya mundo osuan da famosua), beti gure onduan egotiagatik. Badakitzu errekabarrenen beste familixa bat daukotzula. Patxi ta Susanai, milesker edozertan laguntzeko prest egotiagatik eta domekako bazkaixak animetiagatik. Julianzin! Eskerrik asko goixak zure berriketaldixaz piztiagatik, kotileo moment-engatik, nire tesiko

gorabeherak entzutiagatik…Nire tio ta tiatxo danei, nire primo ta primei (larreiko zare eta ez jat zuen izen danak hemen kabiruten! ;)) ta euren seme alabatxo maitiei…(honeik bai kabiruten die!jaja) Harkaitz, Eider, Deba, Ekaitz, Arhane, Danel, Haizea ta Enaitz. Eskerrik asko zuekin egotiakin bakarrik edozein problema ahaztaraztiagatik. Asko gure zauiet! Xaladito batzuk zare!

Eskerrik asko danoi!

ACKNOWLEDGEMENTS FOR FINANCIAL SUPPORT

This thesis has been partially supported by the Basque Government (Consolidated Groups, IT-407-07) and the University of The Basque Country (UPV/EHU) (UFI 11/32). Ane Garate gratefully acknowledge the support provided by the Baque Government for the fellowship grant.

ACKNOWLEDGEMENTS TO THE EDITORIALS

Authors would like to thank the editorials for granting permission to reuse their previously published articles in this thesis:

The links to the final published versions are the following:

Garate *et al.* Therapeutic Delivery 3 (6), 761-774 (2012). http://www.future-science.com/doi/pdf/10.4155/tde.12.53

Garate *et al.* J Drug Target. 27, 1-7 (2015). http://informahealthcare.com/doi/abs/10.3109/1061186X.2015.1020428

Santos *et al.* J Biomed Mater Res A. 102 (11), 3965-3972 (2014). http://onlinelibrary.wiley.com/doi/10.1002/jbm.a.35073/abstract

Ainhoantzat

...y te prometo que aunque te entre el
logure, hay unos duendecillos en la noche
que a partir de una hora te reponen las
pilas y vuelves a despertar...

Glossary

AD: alzheimer's disease
ADSC: adipose derived stem cells
APA: alginate-poly-L-lysine-alginate microcapsules
BFGF: basic fibroblast growth factor
BHK: baby hamster kidney
BMP-2: bone morphogenetic protein 2
BMSC: bone marrow stromal cells/ bone marrow stem cells
BrdU: bromodeoxyuridine
CaCl2: calcium chloride
CaCO3: calcium carbonate
CaSO4 : calcium sulfate
CNS: central nervous system
CPC: calcium phosphate cements
D1-MSC: D1 mesenchymal stem cells
DMEM: dulbecco's modified eagle growth medium
DNA: deoxyribonucleic acid
DPBS: dulbecco's phosphate-buffered saline
DS: degree of substitution
ECM: extracellular matrix
EDC: (1-ethyl-dimethylaminopropyl) carbodiimide
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
ELISA: enzyme-linked immunoabsorbent assay
EPO: erythropoietin
ESC: embryonic stem cells
FBS: faetal bovine serum
FGF2: fibroblast growth factor
FIX: factor IX
GLP-1: glucagon like peptide-1

HA: hialuronic acid
HCl: hydrochloric acid
HGF: hepatocyte growth factor
HOP: human osteoprogenitors
IGF-1: insulin-like growth factor-1
IKVAV: isoleucine-lysine -valine-alanine-valine
IL: interleukin
IPC: insulin-producing cells
mAb: monoclonal antibodies
MFI: mean fluorescent intensity
MMPs: matrix metalloproteainases
MSC: mesenchymal stem cells
NPCs: neural progenitor cell
NSC: neural stem cells
NTF: neurotrophic factors
PBS: phosphate-buffered saline
PDGF: platelet-derived growth factor
PEG: poly(ethylene glycol)
PLGA: poly(lactic-co-glycolic) acid
PLL: poly-L-lysine
PRP: platelet rich plasma
RGD: arginine-glycine-aspartate
SDF-1: stromal cell-derived factor
SPIO: superparamagnetic iron oxide
TCP: tricalcium phosphate
TGF-β: transforming growth factor-β
UPLVG: ultra pure low-viscosity high guluronic acid alginate
VEFG: vascular endothelial growth factor
YIGSR: tyrosine-isoleucine-glycine-serine-arginine

Index

1. Hypothesis 1

2. Introduction 7

Stem cells in alginate bioscaffolds 11

3. Objectives 49

4. Experimental design 53

Chapter 1: Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems 57

Chapter 2: The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells. *In vitro* and *in vivo* direct comparative study 77

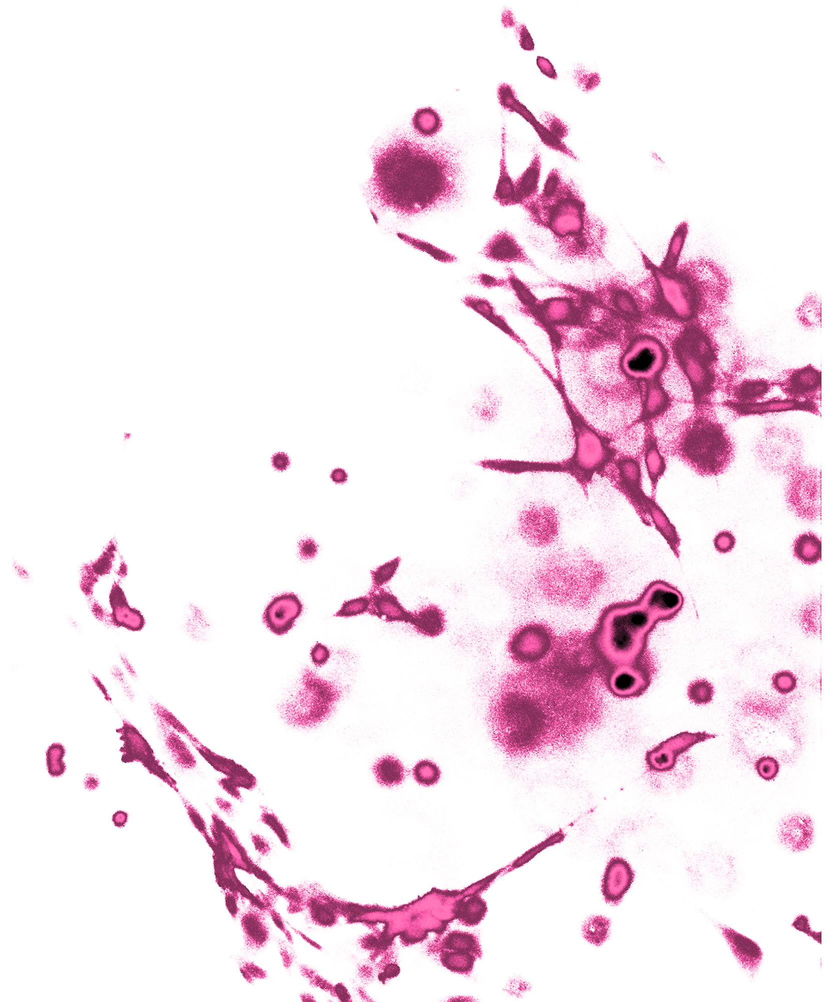
Chapter 3: Assessment of the behavior of MSCs immobilized in biomimetic alginate microcapsules 99

5. Discussion 123

6. Conclusions 147

7. Bibliography 151

Hypothesis



In the last decades, cell based biological systems are evolving rapidly achieving more sophisticated devices for cell immobilization. Biomaterial strategies are gaining more attention in the scientific community to obtain increasingly biological scaffolds leaving behind the traditional inert platforms [1,2].

Among the wide range of possibilities that biomaterial science offers, the chance of including bioactive molecules which provide attachment to immobilized cells is particularly relevant. Indeed, some mammalian cells need adhesion moieties to survive into 3D scaffolds and the incorporation of extracellular matrix (ECM) proteins on cell matrices represents a suitable strategy to overcome this drawback [3,4]. Besides the long natural components of ECM as fibronectin or collagen, other small molecules derived from these proteins have also been employed for those purposes [5]. Probably (Arginine-Glicine-Aspartate) RGD is one of the most frequently employed small proteins and according to the existing literature, it is highly effective at promoting the attachment of numerous cell types [6].

However, recent investigations have shed controversial results concerning the effect of this adhesion moiety, opening an extended debate about its use [7-9]. While *in vitro* studies have confirmed the effectiveness of RGD peptides in enhancing cell function through the regulation of integrin-mediated signaling pathway, *in vivo* studies have been shown to be more variable [10-12]. The background produced by the serum proteins adsorbed in the matrix [13,14] or the synergistic effect mediated by the different physicochemical cues coming from the surrounded microenvironment [15,16] are some factors that could influence on this lack of consistency.

Cell microencapsulation technology is a well-known cell-based therapy which allows the implantation of genetically modified non-autologous cells with the aim of achieving a sustained secretion of therapeutic factors. These cells are normally immobilized into polymeric matrices surrounded by a semipermeable membrane to avoid the host immune response, as in the case of alginate-poly-l-lysine-alginate (APA) microcapsules [17,18]. Preserving the viability of encapsulated cells is of crucial importance to achieve a suitable drug delivery system, since the reduction of living cells may come along with the decrease of therapeutic factor secretion.

Assuming this, we hypothesized that the inclusion of the small tripeptide RGD derived from longer proteins of ECM as bioactive molecule into alginate microcapsules might improve encapsulated cell survival, increasing their long-term viability and thus, maintaining our drug delivery devices functional for longer. Nevertheless, some variables which could vary RGD effectiveness must be taken into account in the design of RGD-modified scaffolds, including RGD density, one of the main parameters that should be defined for each particular cell type [19,20]. We decided to evaluate the influence of the same alginate matrices modified with low, intermediate or high RGD densities in different cell types to observe if the optimal adhesion ligand density changes depending on this factor.

Fibroblasts and myoblasts have been widely employed in cell-based therapies and diverse works have investigated their behavior in RGD modified scaffolds, mainly focused in regenerative medicine applications [21-23]. However, the effect of RGD-modified alginate on these cells has not been thoroughly analyzed for drug delivery applications and there we considered exploring further the effects of RGD density on cell functionality within 3D microcapsules.

Nevertheless, over the years, cell biology also has suffered an evolution: the emergence of stem cells with their impressive biological characteristics has been a turning point for cell based therapies increasing our desire to use these promising cells for drug delivery systems [24-26]. Thus, we decided to evaluate our RGD-modified scaffolds also with therapeutic factor secreting D1 mesenchymal stem cells (MSCs) to analyze their behavior in our matrices and to prove if they would be a suitable cell type for drug delivery devices. The present doctoral thesis aims to provide new insights on the biomimetization of alginate 3D matrices with RGD and how RGD density may impact cell functionality and drug delivery.

References

1 Santos E, Hernandez RM, Pedraz JL, Orive G. Novel advances in the design of three-dimensional bio-scaffolds to control cell fate: translation from 2D to 3D. Trends Biotechnol. 30, 331-341 (2012).

2 Hernandez RM, Orive G, Murua A, Pedraz JL. Microcapsules and microcarriers for in situ cell delivery. Adv.Drug Deliv.Rev. 62, 711-730 (2010).

3 Stupack DG, Cheresh DA. Get a ligand, get a life: integrins, signaling and cell survival. J.Cell.Sci. 115, 3729-3738 (2002).

4 D'Souza SE, Ginsberg MH, Plow EF. Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. Trends Biochem.Sci. 16, 246-250 (1991).

5 Barker TH. The role of ECM proteins and protein fragments in guiding cell behavior in regenerative medicine. Biomaterials 32, 4211-4214 (2011).

6 Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 24, 4385-4415 (2003).

7 Bellis SL. Advantages of RGD peptides for directing cell association with biomaterials. Biomaterials 32, 4205-4210 (2011).

8 Collier JH, Segura T. Evolving the use of peptides as components of biomaterials. Biomaterials 32, 4198-4204 (2011).

9 Williams DF. The role of short synthetic adhesion peptides in regenerative medicine; the debate. Biomaterials 32, 4195-4197 (2011).

10 Alsberg E, Anderson KW, Albeiruti A, Rowley JA, Mooney DJ. Engineering growing tissues. Proc. Natl.Acad.Sci.U.S.A. 99, 12025-12030 (2002).

11 Eid K, Chen E, Griffith L, Glowacki J. Effect of RGD coating on osteocompatibility of PLGA-polymer disks in a rat tibial wound. J.Biomed.Mater.Res. 57, 224-231 (2001).

12 Ferris DM, Moodie GD, Dimond PM, Gioranni CW, Ehrlich MG, Valentini RF. RGD-coated titanium implants stimulate increased bone formation *in vivo*. Biomaterials 20, 2323-2331 (1999).

13 Allen LT, Tosetto M, Miller IS, et al. Surface-induced changes in protein adsorption and implications for cellular phenotypic responses to surface interaction. Biomaterials 27, 3096-3108 (2006).

14 Roach P, Farrar D, Perry CC. Surface tailoring for controlled protein adsorption: effect of

topography at the nanometer scale and chemistry. J.Am.Chem.Soc. 128, 3939-3945 (2006).

15 Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. J.Biol.Chem. 264, 380-388 (1989).

16 Sinha RK, Tuan RS. Regulation of human osteoblast integrin expression by orthopedic implant materials. Bone 18, 451-457 (1996).

17 Acarregui A, Murua A, Pedraz JL, Orive G, Hernandez RM. A perspective on bioactive cell microencapsulation. BioDrugs 26, 283-301 (2012).

18 Murua A, Orive G, Hernandez RM, Pedraz JL. Xenogeneic transplantation of erythropoietin-secreting cells immobilized in microcapsules using transient immunosuppression. J.Control.Release 137, 174-178 (2009).

19 Rowley JA, Madlambayan G, Mooney DJ. Alginate hydrogels as synthetic extracellular matrix materials. Biomaterials 20, 45-53 (1999).

20 Koo LY, Irvine DJ, Mayes AM, Lauffenburger DA, Griffith LG. Co-regulation of cell adhesion by nanoscale RGD organization and mechanical stimulus. J.Cell.Sci. 115, 1423-1433 (2002).

21 Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. J.Biomed.Mater.Res. 60, 217-223 (2002).

22 Dai W, Saltzman WM. Fibroblast aggregation by suspension with conjugates of poly(ethylene glycol) and RGD. Biotechnol.Bioeng. 50, 349-356 (1996).

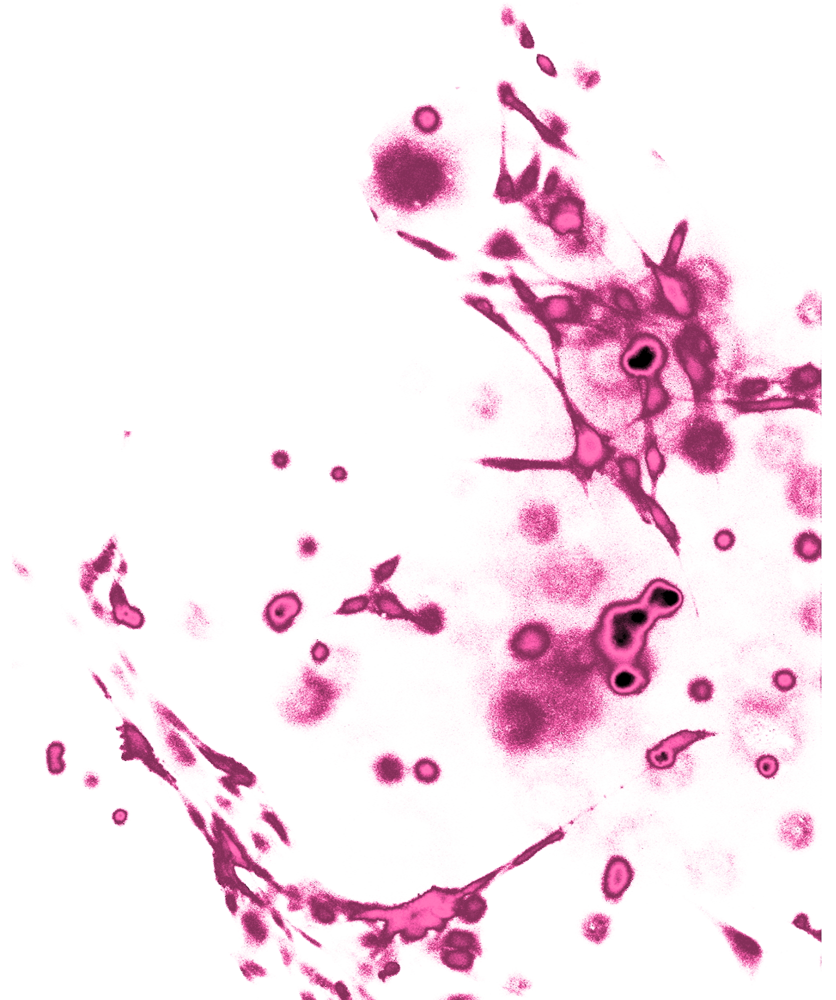
23 Jiang LY, Lv B, Luo Y. The effects of an RGD-PAMAM dendrimer conjugate in 3D spheroid culture on cell proliferation, expression and aggregation. Biomaterials 34, 2665-2673 (2013).

24 Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood 105, 2214-2219 (2005).

25 Boomsma RA, Geenen DL. Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. PLoS One 7, e35685 (2012).

26 Chen PM, Yen ML, Liu KJ, Sytwu HK, Yen BL. Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells. J.Biomed.Sci. 18, 49-0127-18-49 (2011).

Introduction



Stem cells in alginate bioscaffolds

Therapeutic Delivery 3 (6), 761-774 (2012)

Stem cells in alginate bioscaffolds

Ane Garate[1,2], Ainhoa Murua[1,2], Gorka Orive[1,2], Rosa María Hernández[1,2], José Luis Pedraz[1,2]

1NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Vitoria, Spain.
2Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria, Spain.

ABSTRACT

The immobilization of cells into polymeric scaffolds releasing therapeutic factors, such as alginate microcapsules, has been widely employed as a drug-delivery system for numerous diseases for many years. Alginate is the biomaterial of choice for the elaboration of diverse biomimetic scaffolds due to its excellent properties. Additionally, during recent decades, stem cells have gained the attention of the scientific community in the field of cell microencapsulation technology and have opened many perspectives. These cells represent an ideal tool for cell immobilization and so does alginate offering us the opportunity of benefiting from both disciplines in a synergistic way. This review intends to give an overview of the many possibilities and the current situation of immobilized stem cells in alginate bioscaffolds, showing the diverse therapeutic applications they can already be employed in; not only drug-delivery systems, but also tissue engineering platforms.

Keywords
Cell encapsulation, alginate, cell therapy, drug delivery, regenerative medicine, scaffold, stem cells, stem cell fate

Table of contents

1. Background

2. Alginate as a polymeric matrix for cell microencapsulation

3. Stem cells and alginate: the influence of the polymer on stem cell fate

4. Dual activity of encapsulated stem cells: from tissue regeneration to continuous growth factor delivery

5. Applications of encapsulated therapeutic stem cells

- 5.1. Type 1 diabetes
- 5.2. Cancer and Hemophilia
- 5.3. Cartilage repair
- 5.4. Bone tissue regeneration
- 5.5. Heart diseases
- 5.6. Central Nervous System (CNS)
- 5.7. Other organs

6. Conclusion and future perspective

1. Background

In recent decades, the immobilization of cells into polymeric bioscaffolds to release therapeutic factors has been widely employed like drug-delivery systems in numerous diseases [1-4]. Since 1980, when Lim and Sun encapsulated islets of Langerhans as bioartificial endocrine pancreas [5] the use of this type of cell-entrapping system and, more precisely, alginate-poly-L-lysine-alginate (APA) microcapsules, were promoted, making this polymeric system an attractive new therapeutic tool. The immobilization of cells in alginate surrounded by a semipermeable membrane allows the bidirectional diffusion of nutrients, oxygen, therapeutic products and waste of enclosed cells while at the same time prevents the entrance of immune cells and antibodies, permitting a safe transplantation of encapsulated cells without the use of immunosuppressive therapies. This type of alginate scaffold is one of the most widely studied devices for the immunoprotection of transplanted therapeutic cells [6-10].

Based on the potential alginate offers as a tunable biomaterial together with the stem cells advantages, many types of alginate-based bioscaffolds are currently being developed, not only for drug-delivery purposes, but also to be used as devices for applications in regenerative medicine [11-13]. This review comprises an overview of how the combined use of alginate scaffolds and stem cells can benefit from each other, as well as the latest therapeutic approaches in which these cell-based devices are being employed (i.e., drug-delivery systems, tissue engineering and regenerative medicine).

2. Alginate as a polymeric matrix for cell microencapsulation

Alginate is a negatively charged natural polymer typically obtained from brown seaweed and composed of (1-4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) [14]. Alginates with high G content result in more stable gels of greater permeability than those with high M content. On the other hand, M residues are known to provide elasticity to the gel so the mechanical properties of alginate are influenced by the ratio of G and M blocks [15,16]. Importantly, alginate can be purified to a very high purity and did not induce any significant foreign-body

reaction when implanted into animals [17]. The biocompatibility and low toxicity of this biomaterial makes it an ideal choice for cell encapsulation technology and it has become the most studied material for immobilization of living cells [18,19] .

However, beside previous mentioned advantages, alginate is an inert polymer and does not promote cell attachment which may be a critical condition for high cell viability and proliferation for some cell types [20,21]. Thus, recently alginate derivatives containing cell-adhesive peptides have been gaining significant attraction. The cell binding peptides include native long chain extracellular matrix (ECM) proteins [22]such as gelatin [23], xyloglucan [24], collagen [25], fibronectin [26]or fibrinogen [27], as well as short peptide sequences derived from intact ECM proteins that can incur specific interactions with cell receptors. Various substrates have been functionalized with short peptide moieties such as Tyr-Ile-Gly-Ser-Arg (YIGSR) or Ile-Lys-Val-Ala-Val (IKVAV) [28]. However, probably the most studied short peptide for alginate funtionalization is the sequence Arg-Gly-Asp (RGD), the signaling domain derived from fibronectin and laminin. This peptide sequence have been extensively used as adhesion ligand to promote cell attachment and to regulate cellular interactions in alginate scaffolds prolonging cell survival in these non-cell-interactive matrices [29,30].

The versatility of alginate as a biopolymer also offers other properties which may have a great impact on the encapsulated cells. Encapsulation of alginate is generally carried out by dripping a suspension of cells and sodium alginate into a solution of $CaCl_2$ due to the ionic crosslinking between alginate and divalent cations. Although calcium is the cation most commonly used to induce alginate gel formation including $CaSO_4$ or $CaCO_3$, barium ions show higher affinity towards alginate, increasing the alginate´s mechanical stability [31]. However, long gelation times, which are essential for the generation of uniformly crosslinked barium-alginate spheres, are not feasible considering the toxicity of barium ions [32]. The structure of alginate within microcapsules depends not only on the type and concentrations of cations but also on the process of ionic linkage formation.

These parameters together with alginate composition mentioned above or its biofunctionalization by adhesion

motifs are of significant importance when immobilizing cells [33]. FIGURE 1 collects some microcapsule properties which can be of outstanding importance in cell microencapsulation systems.

3. Stem cells and alginate: the influence of the polymer on stem cell fate

In the last years, the use of stem cells has produced a great impact in the field of cell microencapsulation, providing potential benefits that other cell types cannot offer so far. For example, the possibility of differentiation into diverse cell lineages [34]. Moreover, mesenchymal stem cells (MSC) are being carefully studied and considered due to the highly interesting features they present. In addition to the low immunogenicity [35], which turns out to be a feature of outstanding importance in the field of transplantation of organs, tissues and/or cells, they are able to secrete a variety of therapeutically

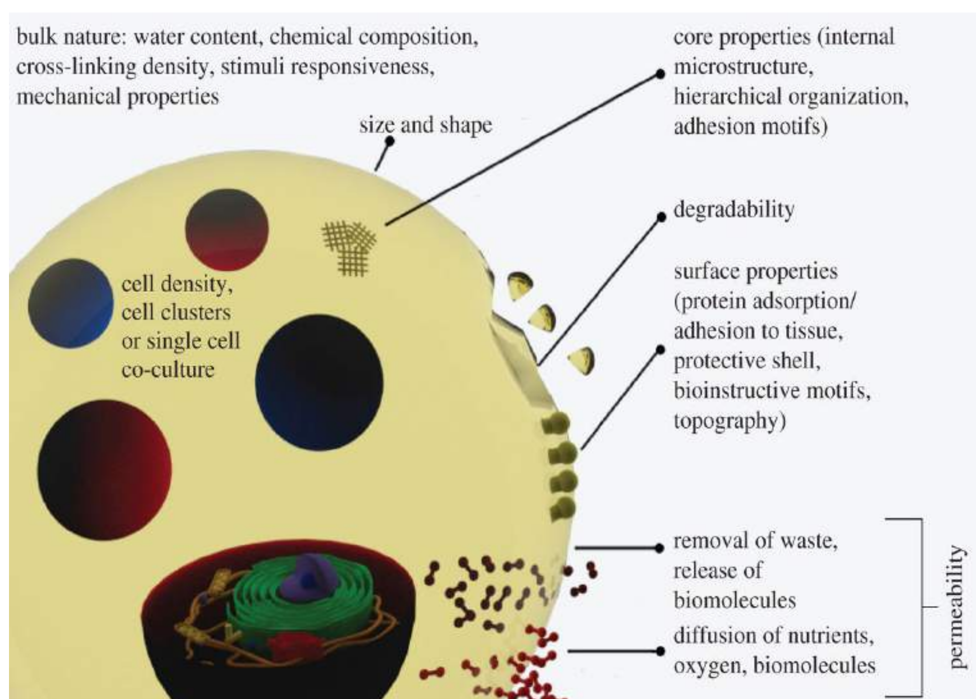


Figure 1. A scheme outlining the properties of microcapsules for cell encapsulation. Reprinted with permission from [19]. © The Royal Society (2014).

amenable cytokines and growth factors spontaneously [36,37].

The use of stem cells combined to adaptable biomaterials, such as alginate, has opened many perspectives in the field of cell microencapsulation. Apart from their applicability as drug-delivery systems [38], stem cell immobilization in alginate biomatrices is also being employed for tissue regeneration due to many characteristics they show, for example, differentiation to specific cell lineages or integration in the implantation site [39,40].

The biomaterial employed in the development of a bioscaffold can change the behavior of stem cells, either in terms of viability, proliferation or differentiation [34,41]. There are a variety of porous scaffold polymers, such as alginate [42], collagen [43], calcium phosphate [44] or polyethylene glycol (PEG) [45,46], but nowadays alginate is the biomaterial of choice due to the fact that it shows the most pliant characteristics for many therapeutic applications [47-49].

Focusing on alginate biosystems, either encapsulated or in hydrogels [42,50], by varying different parameters during the elaboration process, such as cell density, alginate types, divalent cation concentrations, PLL concentration (when preparing APA bioscaffolds) or even diameter of the bead employed, stem cell fate might be controlled [51-55]. Hence, it can be said that the way 3D platforms are developed will have an influence on cell mechanobiology and fate [56].

Among other factors, the elasticity of the niche where cells are entrapped has a great influence on their behavior [57]. As shown in FIGURE 2, the fate of a multipotent stem cell may be determined by the flexibility of the environment. Arrays of long pillars are, therefore, effectively equivalent to a soft substrate (perhaps as soft as fat), whereas arrays of short pillars might be perceived by cells as effectively stiff or rigid, like bone. Cells possess tactile mechanism

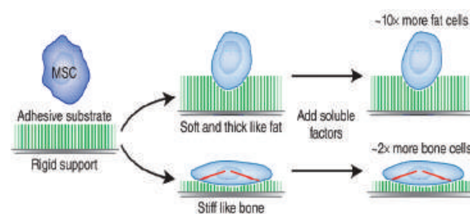


Figure 2. Adherent stem cells need to attach to a solid substrate to survive, but whether the cells attach to a substrate that is soft or one that is stiff can influence differentiation. Reprinted with permission, from [56] ©Macmillan Publishers Ltd (Nature Methods) (2010).

to allow them to feel differences and, in some cases, the factor that can produce the differentiation of stem cells is the elasticity of their microenvironment [58,59].

Another interesting modification alginates offer which can influence the behavior of stem cells is the previous mentioned functionalization with ECM proteins or their derivatives. In addition to its influence on cell bioactivity, adhesion moieties as RGD also promotes a higher differentiation capacity of MSCs. Many research groups are making great efforts in this promising therapeutic field, which is in demand by the scientific community due to the lack of therapeutic alternatives to treat tissue engineering problems [60,61].

Focusing on tissue regeneration using stem cells, the nature of growth-factor delivery systems plays a significant role in regulating stem cell behavior, because stem cell differentiation into a specific lineage involves a cascade of the multiple events with which certain growth factors or hormones are temporally and spatially associated [62,63].

In addition to locating the cells into the injury site, these scaffolds can provide, or be cultured with, different factors (e.g., growth factors, cytokines or inducer factors) that control stem cell fate [64]. An interesting work recently reported by Li *et al.* showed that they were able to differentiate embryonic stem cells (ESCs) into hepatocytes and neuronal cell lineages by changing the alginate concentration of microcapsules, and then culturing them in the presence or absence of retinoic acid (a soluble inducer of neural cell lineages). Their studies corroborated that the incorporation of retinoic acid into the permeable microcapsule system decreased cell aggregation, enhancing neural-lineage differentiation [65].

4. Dual activity of encapsulated stem cells: from tissue regeneration to continuous growth factor delivery

Regenerative medicine involves the process of creating living, functional tissues to repair or replace those lost one and/or restore organ function due to age, disease, damage or congenital defects [66-68]. In certain regenerative medicine applications, it is desirable to use degradable scaffolds for fast release of therapeutic products from the entrapped cells in the injury site. Alginate,

being a tailorable polymer which can be modulated depending on the intended application [69,70] also offers the possibility to modify its degradation rate. Partial oxidation and bimodal molecular-weight distribution are two strategies that have been successfully combined to regulate alginate gel degradation [71].

Using cell microencapsulation as a biotechnological approach, cells may be conducted to a specific organ to become integrated in the new microenvironment [72,73]. As previously mentioned, the type of scaffold plays a key role in the fate of stem cells, but in addition, depending on the type of stem cell selected, such as MSCs [73], ESCs [74], NSCs [75] and/or adipose-derived stem cells (ADSC) [76], the final outcome will also be different (FIGURE 3).

In addition to drug-delivery applications, the use of stem cells in tissue regeneration is a promising therapeutic tool in many fields such as cartilage [77], bone [78], myocardial [79], renal [80] or neuronal repair [81], among others, and depending

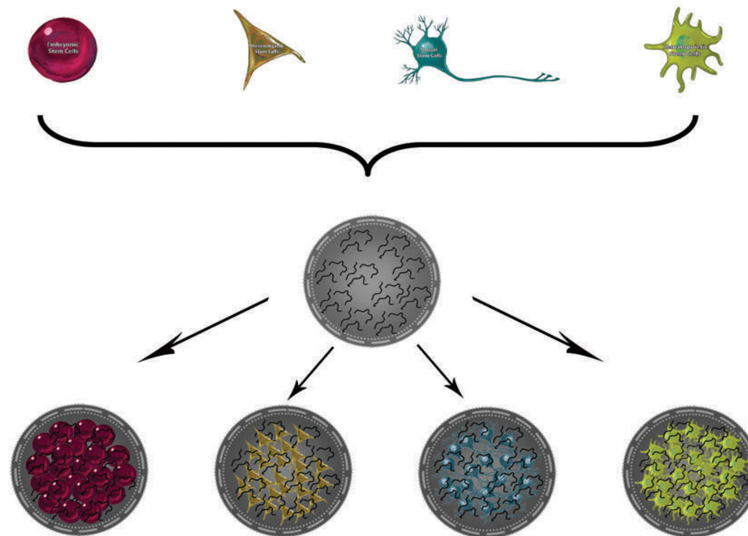


Figure 3. Combination of alginate scaffolds and stem cells. A new therapeutic tool not only for drug delivery, but also regenerative medicine and tissue engineering. The influence of alginate over stem cell fate.

on the injury site, different types of stem cells are used. As previously mentioned, due to their potential benefits and interesting characteristics, one of the most frequently employed stem cells in tissue engineering and regenerative medicine are MSCs.

It has been reported that MSCs can secrete many growth factors, including VEGF [82], HGF [83], IGF-1 [84], SDF-1 [85], BFGF [86], matrix metalloproteainases (MMPs) [87], TGF-β [88] and PDGF [89]. These growth factors and cytokines stimulate endogenous repair mechanisms [90]. For example, various growth factors have been found to be of outstanding importance when infarct occurs, such as VEGF, PDGF or FGF2, all of them being key molecules in blood vessel formation [91]. Moreover, depending on the application, these cells also can be genetically modified for the secretion of diverse growth factors [92] or other therapeutic products [93,94]. In a recently published work, bone marrow stromal cells (BMSCs) were genetically modified to secrete BMP-2 or VEGF followed by alginate encapsulation to induce osteogenic differentiation of autologous nonmodified BMSCs cells. Encapsulated and genetically modified BMSCs were able to secrete a sufficient amount of BMP-2 and VEGF to induce osteogenic differentiation of noonmodified BMSCs. These cells were seeded onto tricalcium phosphate (TCP) scaffolds for *in vivo* implantation to repair orbital wall bone defects in a canine model achieving the bone repair of the orbital wall defect [92].

Another interesting strategy is the possibility to combining the benefits stem cells offer with the advantages of the scaffold in which they are entrapped. Yu *et al.* for example observed an increase in FGF2 expression of human MSCs (hMSCs) encapsulated in RGD-modified alginate compared with nonmodified alginate [95].

The delivery of above mentioned growth factors can induce stem cell differentiation and, thus, create the desired microenvironment for the regeneration of tissues [96]. In a recent work Razavi *et al.* combined those characteristics to promote neurogenic differentiation of stem cells. In their study, adipose derived stem cells (ADSCs) induced to secrete neurotrophic factors (NTF) were encapsulated in alginate microbeads to coculture with non-induced ADSCs evaluating their differentiation potential. They could prove that induced ADSCs were

able to secrete a range of neurotrophic factors whose levels in culture could promote neuronal differentiation of ADSCs [97]. Kerby *et al.* carried out another interesting work co-encapsulating isltes with MSCs. In their *in vivo* study syngenic mouse islets alone or co-encapsulated with MSC were transplanted intraperitoneally into diabetic mice. The results showed that capsules recovered at 6 weeks had greater glucose-stimulated insulin secretion and insulin content in the islet co-encapsulated group. This positive effect was attributed to the immunosuppressive effect mediated by cytokine or metalloproteinase secretion from MSCs [98].

One final therapeutic strategy that the authors would like to mention related to promoting stem cell differentiation, is the use of combined systems composed of encapsulated cells and drug-loaded micro/nano particles [99]. One example is the use of poly (lactic-co-glycolic acid) (PLGA) microparticles containing BMP-2, an osteogenic differentiation growth factor, and dexamethasone (Dex) for bone tissue engineering inside the alginate domain [100]. The release of these two products has been found to promote osteogenic differentiation of MSCs.

The combination of stem cells releasing diverse growth factors and alginate scaffolds that promote their differentiation in a specific cellular lineage to finally become integrated in the injury site represents a potential strategy in regenerative medicine [101].

5. Applications of encapsulated therapeutic stem cells

5.1. Type 1 diabetes

At present, pancreas transplantation is one of the most frequently employed therapies of all cell-based treatments to achieve insulin levels controling plasma glucose. The disadvantages related to this therapy, such as the immunosuppression treatments required, the limited and irregular supply of cadaveric donors [102,103] and the increased mortality [104], have promoted the development of alternative therapeutic approaches for this purpose. Recent studies have demonstrated that stem cells, such as ESCs [105], induced pluripotent stem cells [106] or MSCs [107], can differentiate into cells able to secrete insulin and can, therefore, be considered potentially useful for the treatment of Type 1 diabetes.

An interesting approach was recently proposed by Ngoc *et al.* [108]. They differentiated MSCs derived from mouse bone marrow and human umbilical cord blood into insulin-producing cells (IPCs) and encapsulated them in alginate matrices. To evaluate the capability of these cells, encapsulated and nonencapsulated IPCs differentiated from mMSCs and hMSCs were compared in diabetic mice after intraperitoneal injection. Their results, aside from confirming the differentiation potential of MSCs, showed higher treatment efficacy when cells were encapsulated by means of body weight, blood glucose levels and white blood cell count.

In another similar study, Wang *et al.* encapsulated mouse ESCs (mESCs) within alginate capsules and they incubated these alginate beads with the appropriate medium in order to promote differentiation of encapsulated ESC into IPCs. They observed that differentiated ESCs could release insulin upon glucose challenging *in vitro* in a 3D alginate system [109]. In another study carried out by Tuch *et al.*, hESC were encapsulated in alginate and cultured to produce pancreatic progenitors with four sets of medium containing different growth factors and inhibitors, over 12 days. They transplanted these devices into immunodeficient diabetic NOD/SCID mice to achieve mature IPCs (β cells). The results demonstrated that encapsulated human islets transplanted into diabetic mice readily normalized blood glucose levels and also maintained euglycemia for up to 3 months [110].

Another research group evaluated the possibility of encapsulating hESCs at different stages of differentiation. They could observe that encapsulation of undifferentiated hESCs followed by differentiation induction upon encapsulation resulted in the highest viability and differentiation compared to predifferentiated cells. Surprisingly alginate encapsulation resulted in a much stronger differentiation compared to parallel two-dimensional cultures, demonstrating that alginate encapsulation of hESCs and differentiation to islet-cell types provides a potentially translatable treatment option for type 1 diabetes [111].

5.2. Cancer and Hemophilia

Oncogenesis involves an accumulation of somatic mutations that alter the cell´s genotype and phenotype, leading to a

breakdown of mechanisms controlling normal cell growth and differentiation [112].

Many research groups are investigating the possibilities that cell encapsulation offers in this area, based on the potential benefits and advantages that stem cells offer. In the particular case of MSCs for example, it has been observed that they may exhibit potent anti-tumor effects, which may even be enhanced through genetic modifications. Thus, we can introduce and overexpress exogenous target genes for expression/secretion of a desired therapeutic factor for targeted treatment of different cancer types (FIGURE 4) [113]. The encapsulation of this type of cells permits an increase of retention time in the resection cavity, robust tumor-selective migration and a good secretion of anti-tumor proteins [113,114].

The glioblastoma tumor model depends on angiogenesis to grow. These traits make such tumors highly susceptible to inhibition by antiangiogenic agents [115]. Kleinschmidt *et al.* investigated the cerebral cotransplantation of syngeneic glioma cells and encapsulated hMSCs in immunocompetent rats with cerebral glioma. In this case, empty alginate capsules, capsules containing unmodified hMSCs, and capsules containing genetically engineered hMSC to produce the antiangiogenic peptide endostatin were compared [116]. Results demonstrated a significant reduction of tumor growth after coencapsulation of glioma cells with encapsulated hMSC regardless of the endostatin released.

In an interesting approach carried out by Goren *et al.*, MSCs were transduced before encapsulation to express hemopexin-like protein (PEX), a 210-amino acid fragment of human MMP-2. PEX is known for its antimitotic, anti-invasive and anti-angiogenic properties *in vitro* and *in vivo*, inhibiting endothelial cell proliferation and migration and, thus, resulting in anti-tumor properties. They demonstrated that MSCs can be encapsulated in alginate-poly-L-lysine, maintaining their proliferation in the capsule, long-lasting stability and differentiation potential. One of the advantages of encapsulating engineered hMSCs to secrete tumor inhibitors is the long-term expression of those inhibitors, avoiding repeated administrations and overcoming problems, such as their

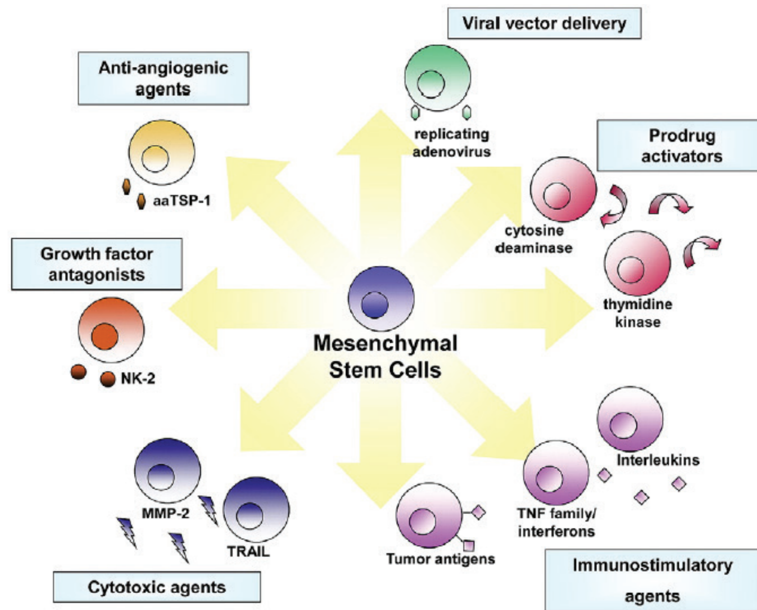


Figure 4. Transgene strategies potentiating MSC for tumor therapy. Tailored to the specific molecular profiles associated with individual tumor types, stem cells can be designed with a variety of different anti-tumor effects. Reproduced with permission, from [113] © Elsevier (2011).

short half-life and instability. In their *in vivo* study, they observed that the injection of hMSC-PEX microcapsules adjacent to the glioma cells of mice, resulted in a suppression in tumor growth, showing a significant decrease in tumor volume and weight [117].

Hemophilia is a genetic disorder related with blood and characterized by the deficiency of plasma proteins required for regular blood clotting. Microencapsulation of cells able to secrete deficient coagulation factors may be a potential strategy to treat this bleeding disorder [94]. Sayyar *et al.* have focused their effort to evaluate the use of alginate based microcapsules to immobilize genetically modified cells able to secrete factor IX (FIX). In a first attempt, they encapsulated FIX-engineered MSCs in fibrinogen-supplemented alginate microcapsules achieving positive results since they found that modified microcapsules significantly increased the viability and proliferation of cells while at the same time enhanced the secretion of FIX compared to non-suplemmented

microcapsules [27]. Following this work, this group also observed the behavior of cord blood derived MSCs in alginate microcapsules modified with RGD or fibronectin. In the first case, the results showed a higher viability in RGD-modified matrices, but a lower level of proliferation and FIX secretion compared to fibronectin supplemented alginate [118]. Additionally, they evaluated the effect of different concentrations of that protein of ECM on the MSCs behavior. They could observe that while low concentration of fibronectin did not significantly affect the viability and protein secretion from the encapsulated cells, higher concentrations improved cell viability, proliferation and FIX secretion [119].

5.3. Cartilage repair

In recent decades, research studies involving cartilage repair in patients of different ages have increased. Specific characteristics of this connective tissue, such as the limited vascular properties and poor capability of regeneration, have impulsed the study of alternative therapies, such as therapeutic approaches based on stem cell immobilization [120].

In addition to the most employed strategies in the field, such as autologous chondrocyte implantation [121], MSCs are also being considered for cartilage repair since MSCs offer several advantages over chondrocytes. Autologous chondrocytes may present some limitations, such as the reduction of chondrogenesis capability with increasing age and the reduced ability to maintain their characteristics with cummulative passages [122]. Moreover, the isolation of this type of cells requires a biopsy of articular cartilage and this can produce additional damage to the joint surface [123]. MSCs have emerged as an attractive alternative for these treatments, not only because of their self-renewal capability and differentiation potential towards chondrogenic lineages, but also because they do not demonstrate the unpleasant disadvantages related to obtention of the cells, as in autologous chodrocyte implantation strategies [124].

An interesting work was focused on define the appropriate cell source to generate a functional and stable matrix. The behavior of human chondrocytes from ear, nose and articular joint as well as bone marrow derived and adipose derived MSCs was evaluated. After culture-expansion and encapsulation in alginate matrices, scaffolds were implanted subcutaneously.

They found that cells from articular joint had the highest chondrogenic capacity *in vitro* while chondrocytes from ear and nose were more potent *in vivo* [125].

In the presence of appropriate growth factors, MSCs also undergo chondrogenesis with consecutive deposits of specific cartilage matrix [126]. One of the growth factors that induce chondrogenesis is TGF-β [127,128]. Besides stimulation of chondrocyte proliferation, experimental studies have shown the influence of this factor in cartilage hypertrophy prevention [129]. However, there are some difficulties when it comes to implanting scaffolds delivering factors such as TGF-β *in vivo* due to scaffold diffusion, foreign-body response and proteolytic activity [130]. There lies the interest in creating a biocompatible scaffold with sustained local delivery of TGF-β to implement MSCs directly into cartilage defect sites [131].

Several works have been conducted to prove MSC differentiation capability into cartilage. For example, an *in vivo* study compared allogenic chondrogenic pre-differentiated MSCs (CMSC) and undifferentiated MSCs for the repair of full thickness articular cartilage defects. In this work, Dashtdar *et al.* created bilateral full thickness cartilage defects on the media femoral condyles of rabbits. They treated defects of the right knee, using the other knee as a control, and worked with two groups of rabbits. One group received alginate-encapsulated MSCs, while the second group received encapsulated CMSCs [132]. To achieve predifferentiation of MSCs, TGF-β3 and BFGF were used. Results demonstrated a production of good quality cartilage in the treated knees without any significant differences between MSCs and CMSCs. Other studies have also reported that alginate capsules can induce chondrogenesis of MSCs without the need of stimulating factors [133]. In a recent work carried out by Valiani *et al.*, the differentiation potential of chondrocytes from ADSCs in alginate/ carbon nano-tubes scaffolds with and without TGF-β1 was investigated. As expected, results showed a higher expression of chondrogenic-related genes, collagen type II and SOX 9 in the group cultured with medium enriched with TGF-β1 [134].

5.4. Bone tissue regeneration

There are diverse injuries, such as congenital malformations, trauma,

skeletal diseases and tumor resections, which require new bone formation. The need for suitable and efficient bone repair is increasing, and this is encouraging tissue engineering advances.

Although many biomaterials have been used in bone formation [135], one of the most recurrent is alginate, due to its intrinsic tunable properties offering many possibilities [20]. It is well known that the natural degradation of alginate, without any modification, is slow and uncontrollable. However, in some cases the objective of the study is the integration of the cells in the damaged tissue. In those cases, the degradation of alginate may be desirable and partial oxidation is one of the most employed method for this purpose [136].

Grellier *et al.* immobilized human osteoprogenitors (HOP) from bone marrow MSCs with or without human umbilical vein endothelial cells inside irradiated, oxidized and/or RGD-grafted alginate microspheres. The aim of this study was to prove the influence of endothelial cells in the osteogenic potential of HOPs in bone regeneration. They saw a significant increase of mineralization when HOPs were co-immobilized together with human umbilical vein endothelial cells, and alginate was modified with RGD [60]. With these results they demonstrated the benefits RGD-modified alginates offer, not only in terms of cell behavior, but also to promote differentiation of cells.

Other groups employed also RGD modified-alginate scaffolds for bone formation. In an interesting study, Bhat *et al.* presented microbeads coated with extracellular matrix (ECM) secreted from MSCs suspended in aginate hydrogels. Human MSCs entrapped in alginate hydrogels containing these cell-interactive beads exhibited higher osteogenic gene expression compared to RGD-modified hydrogels. Bone formation at 6 weeks was similar in both cases, demonstrating that engineered ECM can also be employed to direct formation of bone tissue [137].

Another interesting strategy for bone regeneration is the employment of calcium phosphate cement (CPC), which contains oxidized alginate-fibrin microbeads. These novel degradable microbeads permit the release of human umbilical cord MSCs (hUCMSC) into the CPC scaffolds, while the macropores of the cement permits the maintenance of good viability levels. It could be stated that the

degradation rate in oxidized alginate-fibrin is higher than alginate microbeads without modifications or even than oxidized alginate microbeads [138].

Zhao *et al.* have also developed a similar type of scaffold by modifying CPCs. In this case, injectable and mechanically resistant cell constructs with CPC-paste-containing alginate hydrogels were developed with the aim of protecting hUCMSC during the mixing and injection procedures, to avoid any stress the CPC paste may provoke on the cells. Moreover, to improve mechanical properties of CPCs, they used chitosan and absorbable suture fibers and proved that the incorporation of these two products resulted in stronger and tougher scaffolds, without compromising the injectability of the solutions. But most importantly, they demonstrated that the viability of hUCMSCs was not affected and they could be differentiated into the osteogenic lineage after their genetic modification to produce alcalin phosphatase, osteocalcin and collagen I, and express osterix (FIGURE 5) [139].

In a similar approach, Zhou *et al.* compared alginate, oxidized alginate and oxidized alginate-fibrin microbeads encapsulating hUCMSCs. They found

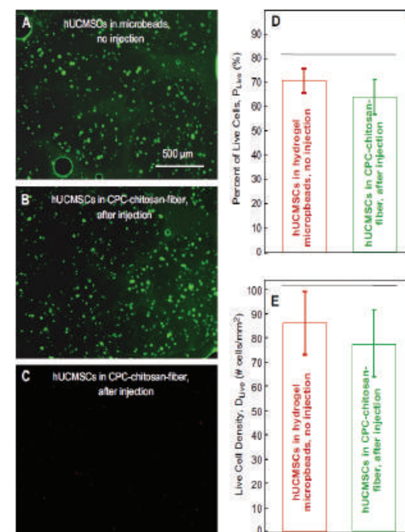


Figure 5. (A) hUCMSCs in microbeads (without CPC, without injection). (B) hUCMSCs in microbeads after mixing with CPC-chitosan-fiber paste and after injection. Live cells (green) were numerous. (C) Dead cells (red) were very few. (D) Percentage of live cells (mean ± sd; n = 5). (E) Live cell density. The CPC mixing and injection process did not significantly harm the encapsulated hUCMSCs. Reprinted with permission from Ref. [139] © Elsevier (2010).

quick degradation of the scaffold and release of the cells from alginate-fibrin microbeads; the released cells also showed excellent proliferation and osteogenic differentiation in addition to synthesizing bone minerals [140]. They proved the great potential of fast-degradable alginate-fibrin microbeads in the release of cells into the injury site.

Huang *et al.* aimed at evaluating the beneficial characteristic that platelet rich plasma (PRP) offers due to the fact that this plasma has several growth factors, such as PDGF, TGF β, BFGF, IGF, VEGF and EGF, which induce osteogenic differentiation of skeletal muscle satellite cells. The aim of the study was to evaluate ectopic bone formation after implantation in the subcutaneous pockets of nude mice of an alginate hydrogel containing muscle satellite cells only or cells in combination with PRP. Their study demonstrated the use of PRP-enhanced induction into the osteoblastic phenotype [141].

5.5. Heart diseases

Heart diseases are major causes of morbidity and mortality linked to extensive loss of cardiac cells. Due to the lack of donor organs for transplantation, cardiac cell replacement therapy has emerged as an appealing alternative to achieve functional cardiomyocytes [142].

When myocardial infarction occurs there is a damage of the infarcted area, and cell therapy is used to repair the injured myocardium [143], but if these cells are injected in the injury site, a large proportion can be lost within the first minutes post-injection due to beating of the heart [144,145].

To address this problem, Al Kindi *et al.* proposed the encapsulation of MSCs in alginate microcapsules to increase the initial retention of the injected cells [146]. Animal studies and clinical trials showed that MSCs could potentially improve ventricular function following ischemic injury using this retention strategy by means of microencapsulating the cells, rather than just administering the naked cells [147]. Additionally, MSCs have also been found to present protective paracrine effects on cardiomyocytes [148], and their ability to induce myocardial regeneration and improve cardiac function has also been demonstrated [149]. Following this approach another group developed a cell encapsulation system which consisted on porcine adipose tissue-derived stem cells (ADSCs) encapsulated in APA microcapsules labeled with superparamagnetic iron oxide (SPIO) to follow cells *in vivo*. After the implantation of non-encapsulated and encapsulated ADSCs in a porcine model of myocardial infarction they proved that cell retention was enhanced after enclosing ADSCs within labeled capsules. However, *in vivo* results also showed

no statistically significant differences in heart rate and cardiac output between treatment groups. In another similar work hMSCs were encapsulated in alginate with the aim of improving cell retention. The intrapericardial delivery of microcapsules were performed safely leading to high cell retention and survival in an immunocompetent swine model [150].

Taking into account the cardioprotective effects of glucagon-like peptide-1 (GLP-1), a research group immortalized and engineered hMSCs to produce a GLP-1 fusion protein to encapsulate in alginate beads. A pig myocardial injury model was used to prove that encapsulated genetically modified MSCs provided a prolonged supply of GLP-1 and paracrine stem cell factors, which improved left ventricular function and reduced epicardial infarct size. In this case, combined benefits of paracrine stem cell factors and GLP-1 were superior to those of stem cells alone [151].

In an attempt to study the effect of scaffold biomaterials on stem cell fate in the field of cardiac repair, Yu *et al.* encapsulated hMSCs in alginate microspheres modified with RGD, and investigated the effect of this type of scaffold on cell survival, maintenance of left ventricle geometry and preservation of left ventricle function in a rat model of acute myocardial infarction. Results showed beneficial effects in stem cell behavior (e.g., migration, proliferation, differentiation and modulation of cytokines and FGF-2 signaling, one of the major signaling molecules in blood-vessel formation), due to the dynamics of cell-ECM interactions, but mainly demonstrated the effectiveness of RGD-modified alginate microspheres for the delivery of stem cells to myocardium and myocardial repair, reducing the infracted area and enhancing arteriole formation [95].

Jing *et al.* explored the cardiogenic potential of mESCs and hESCs encapsulated in alginate poly-L-lysine capsules with a liquid core to enable the production of cardiac cells derived from stem cells. They showed, for the first time, that encapsulated hESCs and mESCs could be coaxed to cardiac cells when cultured in dishes or stirred-suspension vessels [152].

5.6. Central Nervous System (CNS)

The use of strategies based on neural

tissue implantation may contribute to improvements in diseases such as Parkinson´s, Alzheimer´s disease (AD), Huntington´s disease and/or spinal cord injury [153-155]. NSCs are considered very attractive for regenerative purposes because of their ability to self-renew and differentiate into the three major neural lineages (neurons, astrocytes and oligodendrocytes) [156,157]. Moreover, many studies suggest that NSCs have the potential to promote neuroprotection and axonal regeneration of the host tissue [158].

Alginate has been widely employed in the encapsulation of NSCs, not only for the optimal compatibility it shows with central nervous system tissues [159], but also because it allows proliferation, differentiation and integration of newly-formed NSCs as it gradually degrades. Ashton *et al.*, encapsulated alginate lyase into PLGA to degrade alginate enzymatically in a controlled and modifiable fashion; observing a significant increase in the expansion rate of neuronal progenitor cells (NPCs) cultured in alginate that degraded enzymatically, in comparison with NPCs cultured in standard alginate hydrogels. They also demonstrated that PLGA microspheres did not influence NPC proliferation [160].

However, not only NSCs have been encapsulated in alginate matrices to treat central nervious system diseases. Some works present approaches to drive differentiation of ESCs toward neuronal lineages using cell encapsulation in alginate [161]. Bozza *et al.* encapsulated mouse ESCs in alginate beads with or without modification by fibronectin or hialuronic acid (HA). Moreover they also tried to evaluate the influence of the alginate bead stiffness employing different alginate concentrations although all their experimental conditions presented elastic moduli in the range of those found in brain. Their results demonstrated that cell behavior is influenced both by chemical and mechanical properties achieving the most efficient and homogeneous neural differentiation using the lower alginate concentration and modifiying alginate with HA [59]. Banerjee *et al.* also carried out another interesting work focused on the elasticity of alginate to know the influence of alginate elastic modulus on the proliferation and differentiation of encapsulated neural stem cells (NSC). They were able to observe the greatest enhancement in expression of the neural marker β-tubulin III within hydrogels

having an elastic modulus comparable to that of brain tissues [58].

To treat brain injuries, Heile *et al.* proposed an interesting strategy where MSCs were transfected to secrete Glucagon-like peptide-1 (GLP-1), because stimulation of GLP-1 receptors has been found to be associated with neurotrophic activity. Using a controlled cortical impact rat model, they compared hippocampal neuronal cell loss, and neuronal and glial skeletal abnormalities, observing a significant reduction of damage in the cell-treated groups, which was more pronounced in the GLP-1 secreting MSC treated group [162].

Klinge *et al.* employed a similar approach, using the same cell line to study the effects of encapsulated native and GLP-1 transfected MSC in AD. In a double transgenic mouse model of AD, the effects of encapsulated hMSCs with and without GLP-1 delivery on amyloid-β deposition, microglial and glial tissues were investigated after intraventricular implantation of the cell-based devices. They observed a decrease in amyloid-β depositions and suppression of glial and microglial response with both types of encapsulated cells (GLP-1 transfected or not) [163].

Based on the successful results obtained in these studies, Heile *et al.* continued with clinical trials using an intracerebral hemorrhage disease model. They transplanted microencapsulated allogenic hMSCs into the brain tissue cavity after neurosurgical evacuation of the hematoma as shown in FIGURE 6.

Results revealed that up to 30% of the transplanted MSCs survived the 2-week-implantation period and were still secretorily active after explantation. Although the preliminary results suggest that the cell capsules may even decrease cerebral edema, additional preclinical studies are required to determine the biosecurity of the transplantation of cell capsules [164].

5.7. Other organs

At present, liver transplantation is the only effective treatment for terminal liver failure, but it is associated with numerous problems, such as a chronic donor shortage and high costs. However, nowadays there are several groups making great efforts to overcome this issue working on strategies based on tissue engineering and regenerative medicine [165-167].

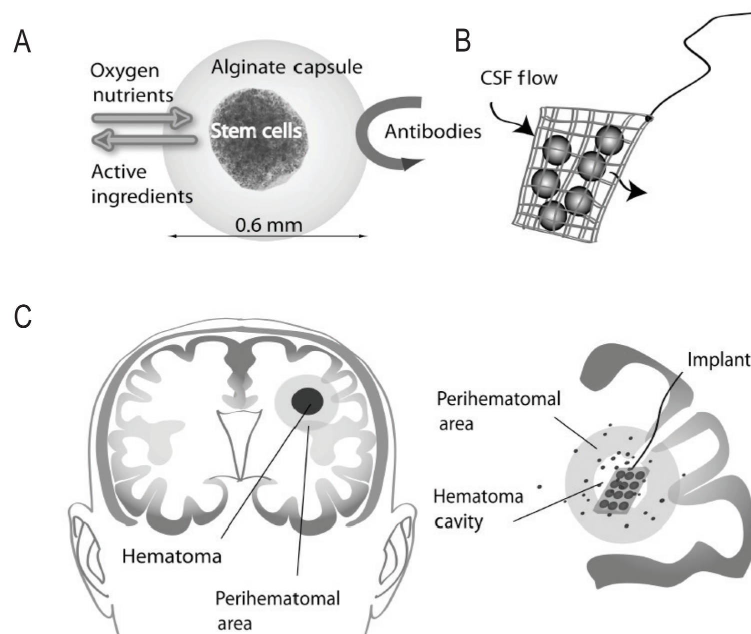


Figure 6. Encapsulated mesenchymal cell biodelivery of GLP-1. (A) Human bone marrow-derived, mesenchymal stem cells producing GLP-1 are encapsulated with alginate. (B) The microcapsules are filled into a 1.5 x 1.5-cm bag that is manually sutured from a polypropylene. (C) The mesh bag is implanted into the hematoma cavity. Reprinted with permission from Dialogues Clin.Neurosci.13, 279-286 (2011) © Les laboratories Servier, Neully-sur-Seine, France [164].

Several research groups have outlined techniques to differentiate stem cells into hepatocytes [168,169]. Maguire *et al.* have proposed the use of APA microcapsules to differentiate mESC into hepatocytes without the formation of embryoid bodies. Their results demonstrate that the alginate microenvironment maintains cell viability, is conductive to ESC differentiation, and maintains the differentiated cellular function [170]. The hepatocytes co-encpasulation with bone marrow stem cells (BMSC) has been the strategy of choice to other reseasrch groups. The *in vivo* studies demonstrated that co-encapsulation of hepatocytes and BMSCs prolongs the viability of hepatocytes and enhances the ability to correct congenital hyperbilirubinemia in rats [171].

In another study regarding renal diseases, Trouche *et al.* implanted MSCs

in APA microcapsules under the renal capsule in a rat model of renal ischemia-perfusion, showing the possibility of grafting microspheres in this particular implantation site with no degradation of the devices and/or impact on renal function but, most importantly, the paracrine activity of MSCs was confirmed, by means of recovery of the kidneys [172].

6. Conclusion and future perspective

Since researchers began using cell encapsulation based on alginate-like drug-delivery system platforms, this technique has developed significantly. Most interestingly, achievements in this field have expanded from drug delivery, to tissue engineering and regenerative medicine applications. The use of stem cells in combination with alginate-based bioscaffolds in cell microencapsulation technology has made this expansion a reality, opening new perspectives.

In this review we have tried to highlight the potential benefits of combining alginate scaffolds and stem cells due to the effect this biomaterial may have on stem cell fate, providing up-to-date information regarding therapeutic applications in the fields of drug delivery, but mainly in tissue engineering and regenerative medicine. Although many works mentioned above still employed non-antilogous stem cells, the impressive biological characteristics of this cell type offer a promising future for cell-based therapies. Besides, the increasingly comfortable resources to obtain stem cells from patients are opening new possibilities in the field allowing the implantation of autologous cells. On the other hand, the modifications of alginate matrices focused on achieving specific goals provide a powerful tool to the research community. Stem cell biology and biomaterial science together are called to be a source of significant advances in the modern society, opening a new era for cell based therapies.

Financial and competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or matterials discussed in the manuscript. This includes employment, consultancies, honorariea, stock ownership or options, expert testimony , grants or patents received

or pending, or royalyies. No writing assistance was utilized in the production of this manuscript.

In memoriam

We would like to thank the contribution of Ainhoa Murua for her invaluable support during all these years. Her extraordinary capacity to understand and promote the research has been of great help for us. Her kind spirit will remain with us forever.

References

1 Borlongan CV, Skinner SJ, Geaney M, Vasconcellos AV, Elliott RB, Emerich DF. Neuroprotection by encapsulated choroid plexus in a rodent model of Huntington's disease. Neuroreport 15, 2521-2525 (2004).

2 Calafiore R, Basta G, Luca G, et al. Standard technical procedures for microencapsulation of human islets for graft into nonimmunosuppressed patients with type 1 diabetes mellitus. Transplant.Proc. 38, 1156-1157 (2006).

3 Hortelano G, Al-Hendy A, Ofosu FA, Chang PL. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. Blood 87, 5095-5103 (1996).

4 Orive G, Anitua E, Pedraz JL, Emerich DF. Biomaterials for promoting brain protection, repair and regeneration. Nat.Rev.Neurosci. 10, 682-692 (2009).

5 Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 210, 908-910 (1980).

6 Tam SK, de Haan BJ, Faas MM, Halle JP, Yahia L, de Vos P. Adsorption of human immunoglobulin to implantable alginate-poly-L-lysine microcapsules: effect of microcapsule composition. J.Biomed.Mater.Res.A. 89, 609-615 (2009).

7 Gugerli R, Cantana E, Heinzen C, von Stockar U, Marison IW. Quantitative study of the production and properties of alginate/poly-L-lysine microcapsules. J.Microencapsul. 19, 571-590 (2002).

8 Orive G, Tam SK, Pedraz JL, Halle JP. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. Biomaterials 27, 3691-3700 (2006).

9 Murua A, Herran E, Orive G, et al. Design of a composite drug delivery system to prolong functionality of cell-based scaffolds. Int.J.Pharm. 407, 142-150 (2011).

10 de Haan BJ, Rossi A, Faas MM, et al. Structural surface changes and inflammatory responses against alginate-based microcapsules after exposure to human peritoneal fluid. J.Biomed. Mater.Res.A. 98, 394-403 (2011).

11 McQuilling JP, Arenas-Herrera J, Childers C, et al. New alginate microcapsule system for angiogenic protein delivery and

immunoisolation of islets for transplantation in the rat omentum pouch. Transplant.Proc. 43, 3262-3264 (2011).

12 Kolambkar YM, Dupont KM, Boerckel JD, et al. An alginate-based hybrid system for growth factor delivery in the functional repair of large bone defects. Biomaterials 32, 65-74 (2011).

13 Popa EG, Gomes ME, Reis RL. Cell delivery systems using alginate--carrageenan hydrogel beads and fibers for regenerative medicine applications. Biomacromolecules 12, 3952-3961 (2011).

14 Remminghorst U, Rehm BH. Bacterial alginates: from biosynthesis to applications. Biotechnol.Lett. 28, 1701-1712 (2006).

15 Drury JL, Dennis RG, Mooney DJ. The tensile properties of alginate hydrogels. Biomaterials 25, 3187-3199 (2004).

16 Martinsen A, Skjak-Braek G, Smidsrod O. Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol. Bioeng. 33, 79-89 (1989).

17 Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog.Polym.Sci. 37, 106-126 (2012).

18 Amsden B, Turner N. Diffusion characteristics of calcium alginate gels. Biotechnol.Bioeng. 65, 605-610 (1999).

19 Gasperini L, Mano JF, Reis RL. Natural polymers for the microencapsulation of cells. J.R.Soc.Interface 11, 20140817 (2014).

20 Kong HJ, Smith MK, Mooney DJ. Designing alginate hydrogels to maintain viability of immobilized cells. Biomaterials 24, 4023-4029 (2003).

21 Mallett AG, Korbutt GS. Alginate modification improves long-term survival and function of transplanted encapsulated islets. Tissue Eng. Part A. 15, 1301-1309 (2009).

22 Mazzitelli S, Luca G, Mancuso F, et al. Production and characterization of engineered alginate-based microparticles containing ECM powder for cell/tissue engineering applications. Acta Biomater. 7, 1050-1062 (2011).

23 Grigore A, Sarker B, Fabry B, Boccaccini AR, Detsch R. Behavior of encapsulated MG-63 cells in RGD and gelatine-modified alginate hydrogels. Tissue Eng.Part A. 20, 2140-2150 (2014).

24 Seo SJ, Akaike T, Choi YJ, Shirakawa M, Kang IK, Cho CS. Alginate microcapsules prepared with xyloglucan as a synthetic extracellular matrix for hepatocyte attachment. Biomaterials 26, 3607-3615 (2005).

25 Tsai S, Jeng M, Tsay R, Wang Y. Gel beads composed of collagen reconstituted in alginate. Biotech Tech 12, 21-23 (1998).

26 Li C, Zhao S, Zhao Y, Qian Y, Li J, Yin Y. Chemically crosslinked alginate porous microcarriers modified with bioactive molecule for expansion of human hepatocellular carcinoma cells. J.Biomed.Mater.Res.B.Appl.

Biomater. 102, 1648-1658 (2014).

27 Sayyar B, Dodd M, Wen J, et al. Encapsulation of factor IX-engineered mesenchymal stem cells in fibrinogen-alginate microcapsules enhances their viability and transgene secretion. J.Tissue Eng. 3, 2041731412462018 (2012).

28 Shin H, Jo S, Mikos AG. Biomimetic materials for tissue engineering. Biomaterials 24, 4353-4364 (2003).

29 Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. J.Biomed.Mater.Res. 60, 217-223 (2002).

30 Hsiong SX, Huebsch N, Fischbach C, Kong HJ, Mooney DJ. Integrin-adhesion ligand bond formation of preosteoblasts and stem cells in three-dimensional RGD presenting matrices. Biomacromolecules 9, 1843-1851 (2008).

31 Morch YA, Donati I, Strand BL, Skjak-Braek G. Effect of Ca2+, Ba2+, and Sr2+ on alginate microbeads. Biomacromolecules 7, 1471-1480 (2006).

32 Krishnamurthy NV, Gimi B. Encapsulated cell grafts to treat cellular deficiencies and dysfunction. Crit.Rev.Biomed.Eng. 39, 473-491 (2011).

33 Huang X, Zhang X, Wang X, Wang C, Tang B. Microenvironment of alginate-based microcapsules for cell culture and tissue engineering. J.Biosci.Bioeng. 114, 1-8 (2012).

34 Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell 126, 677-689 (2006).

35 Chiu RC. MSC immune tolerance in cellular cardiomyoplasty. Semin.Thorac.Cardiovasc. Surg. 20, 115-118 (2008).

36 Park H, Temenoff JS, Holland TA, Tabata Y, Mikos AG. Delivery of TGF-beta1 and chondrocytes via injectable, biodegradable hydrogels for cartilage tissue engineering applications. Biomaterials 26, 7095-7103 (2005).

37 Augello A, Kurth TB, De Bari C. Mesenchymal stem cells: a perspective from *in vitro* cultures to *in vivo* migration and niches. Eur.Cell.Mater. 20, 121-133 (2010).

38 Lee KY, Peters MC, Anderson KW, Mooney DJ. Controlled growth factor release from synthetic extracellular matrices. Nature 408, 998-1000 (2000).

39 Nair A, Shen J, Lotfi P, Ko CY, Zhang CC, Tang L. Biomaterial implants mediate autologous stem cell recruitment in mice. Acta Biomater. 7, 3887-3895 (2011).

40 Salinas CN, Anseth KS. Mesenchymal stem cells for craniofacial tissue regeneration: designing hydrogel delivery vehicles. J.Dent. Res. 88, 681-692 (2009).

41 Spradling Allan, Drummond-Barbosa Daniela, Kai Toshie. Stem cells find their niche.

42 Rowley JA, Madlambayan G, Mooney DJ. Alginate hydrogels as synthetic extracellular

matrix materials. Biomaterials 20, 45-53 (1999).

43 Hui TY, Cheung KM, Cheung WL, Chan D, Chan BP. In vitro chondrogenic differentiation of human mesenchymal stem cells in collagen microspheres: influence of cell seeding density and collagen concentration. Biomaterials 29, 3201-3212 (2008).

44 Moreau JL, Xu HH. Mesenchymal stem cell proliferation and differentiation on an injectable calcium phosphate-chitosan composite scaffold. Biomaterials 30, 2675-2682 (2009).

45 Salinas CN, Anseth KS. The influence of the RGD peptide motif and its contextual presentation in PEG gels on human mesenchymal stem cell viability. J.Tissue Eng. Regen.Med. 2, 296-304 (2008).

46 Salinas CN, Anseth KS. Decorin moieties tethered into PEG networks induce chondrogenesis of human mesenchymal stem cells. J.Biomed.Mater.Res.A. 90, 456-464 (2009).

47 Chayosumrit M, Tuch B, Sidhu K. Alginate microcapsule for propagation and directed differentiation of hESCs to definitive endoderm. Biomaterials 31, 505-514 (2010).

48 Santos E, Zarate J, Orive G, Hernandez RM, Pedraz JL. Biomaterials in cell microencapsulation. Adv.Exp.Med.Biol. 670, 5-21 (2010).

49 Zimmermann H, Zimmermann D, Reuss R, et al. Towards a medically approved technology for alginate-based microcapsules allowing long-term immunoisolated transplantation. J.Mater.Sci.Mater.Med. 16, 491-501 (2005).

50 de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials 27, 5603-5617 (2006).

51 Ma HL, Hung SC, Lin SY, Chen YL, Lo WH. Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. J.Biomed. Mater.Res.A. 64, 273-281 (2003).

52 Tompkins RG, Carter EA, Carlson JD, Yarmush ML. Enzymatic function of alginate immobilized rat hepatocytes. Biotechnol. Bioeng. 31, 11-18 (1988).

53 Shakibaei M, De Souza P. Differentiation of mesenchymal limb bud cells to chondrocytes in alginate beads. Cell Biol.Int. 21, 75-86 (1997).

54 Caterson EJ, Li WJ, Nesti LJ, Albert T, Danielson K, Tuan RS. Polymer/alginate amalgam for cartilage-tissue engineering. Ann.N.Y.Acad.Sci. 961, 134-138 (2002).

55 Kavalkovich KW, Boynton RE, Murphy JM, Barry F. Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system. *In Vitro* Cell.Dev.Biol.Anim. 38, 457-466 (2002).

56 Buxboim A, Discher DE. Stem cells feel the difference. Nat.Methods 7, 695-697 (2010).

57 Candiello J, Singh SS, Task K, Kumta PN, Banerjee I. Early differentiation patterning of mouse embryonic stem cells in response to

variations in alginate substrate stiffness. J.Biol. Eng. 7, 9-1611-7-9 (2013).

58 Banerjee A, Arha M, Choudhary S, et al. The influence of hydrogel modulus on the proliferation and differentiation of encapsulated neural stem cells. Biomaterials 30, 4695-4699 (2009).

59 Bozza A, Coates EE, Incitti T, et al. Neural differentiation of pluripotent cells in 3D alginate-based cultures. Biomaterials 35, 4636-4645 (2014).

60 Grellier M, Granja PL, Fricain JC, et al. The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on mineralization in a bone defect. Biomaterials 30, 3271-3278 (2009).

61 Evangelista MB, Hsiong SX, Fernandes R, et al. Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. Biomaterials 28, 3644-3655 (2007).

62 Karsenty G. The complexities of skeletal biology. Nature 423, 316-318 (2003).

63 Kronenberg HM. Developmental regulation of the growth plate. Nature 423, 332-336 (2003).

64 Benoit DS, Schwartz MP, Durney AR, Anseth KS. Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells. Nat.Mater. 7, 816-823 (2008).

65 Li L, Davidovich AE, Schloss JM, et al. Neural lineage differentiation of embryonic stem cells within alginate microbeads. Biomaterials 32, 4489-4497 (2011).

66 Beltrami AP, Cesselli D, Beltrami CA. Stem cell senescence and regenerative paradigms. Clin.Pharmacol.Ther. 91, 21-29 (2012).

67 Hipp J, Atala A. Tissue engineering, stem cells, cloning, and parthenogenesis: new paradigms for therapy. J.Exp.Clin.Assist.Reprod. 1, 3 (2004).

68 Sumi S. Regenerative medicine for insulin deficiency: creation of pancreatic islets and bioartificial pancreas. J.Hepatobiliary.Pancreat. Sci. 18, 6-12 (2011).

69 King A, Sandler S, Andersson A. The effect of host factors and capsule composition on the cellular overgrowth on implanted alginate capsules. J.Biomed.Mater.Res. 57, 374-383 (2001).

70 Pawar SN, Edgar KJ. Alginate derivatization: A review of chemistry, properties and applications. Biomaterials 33, 3279-3305 (2012).

71 Boontheekul T, Kong HJ, Mooney DJ. Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. Biomaterials 26, 2455-2465 (2005).

72 Facca S, Cortez C, Mendoza-Palomares C, et al. Active multilayered capsules for *in vivo* bone formation. Proc.Natl.Acad.Sci.U.S.A. 107, 3406-

3411 (2010).

73 Bian L, Zhai DY, Tous E, Rai R, Mauck RL, Burdick JA. Enhanced MSC chondrogenesis following delivery of TGF-beta3 from alginate microspheres within hyaluronic acid hydrogels *in vitro* and *in vivo*. Biomaterials 32, 6425-6434 (2011).

74 Bai HY, Chen GA, Mao GH, Song TR, Wang YX. Three step derivation of cartilage like tissue from human embryonic stem cells by 2D-3D sequential culture *in vitro* and further implantation *in vivo* on alginate/PLGA scaffolds. J.Biomed.Mater.Res.A. 94, 539-546 (2010).

75 Seidlits SK, Khaing ZZ, Petersen RR, et al. The effects of hyaluronic acid hydrogels with tunable mechanical properties on neural progenitor cell differentiation. Biomaterials 31, 3930-3940 (2010).

76 Zhu Y, Liu T, Song K, Ning R, Ma X, Cui Z. ADSCs differentiated into cardiomyocytes in cardiac microenvironment. Mol.Cell.Biochem. 324, 117-129 (2009).

77 Mobasheri A, Csaki C, Clutterbuck AL, Rahmanzadeh M, Shakibaei M. Mesenchymal stem cells in connective tissue engineering and regenerative medicine: applications in cartilage repair and osteoarthritis therapy. Histol.Histopathol. 24, 347-366 (2009).

78 Hwang YS, Cho J, Tay F, et al. The use of murine embryonic stem cells, alginate encapsulation, and rotary microgravity bioreactor in bone tissue engineering. Biomaterials 30, 499-507 (2009).

79 Kraehenbuehl TP, Ferreira LS, Hayward AM, et al. Human embryonic stem cell-derived microvascular grafts for cardiac tissue preservation after myocardial infarction. Biomaterials 32, 1102-1109 (2011).

80 Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. Am.J.Physiol.Renal Physiol. 292, F1626-35 (2007).

81 Leipzig ND, Shoichet MS. The effect of substrate stiffness on adult neural stem cell behavior. Biomaterials 30, 6867-6878 (2009).

82 Cai J, Huang Y, Chen X, Xie H, Huang Y, Deng L. Regulation of sonic hedgehog on vascular endothelial growth factor, basic fibroblast growth factor expression and secretion in bone marrow mesenchymal stem cells. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 26, 112-116 (2012).

83 Zhou B, Tsaknakis G, Coldwell KE, et al. A novel function for the haemopoietic supportive murine bone marrow MS-5 mesenchymal stromal cell line in promoting human vasculogenesis and angiogenesis. Br.J.Haematol. (2012).

84 Hsiao ST, Asgari A, Lokmic Z, et al. Comparative Analysis of Paracrine Factor Expression in Human Adult Mesenchymal Stem Cells Derived from Bone Marrow, Adipose, and Dermal Tissue. Stem Cells Dev. (2012).

85 Huang YC, Liu TJ. Mobilization of

mesenchymal stem cells by stromal cell-derived factor-1 released from chitosan/tripolyphosphate/fucoidan nanoparticles. Acta Biomater. 8, 1048-1056 (2012).

86 Tasso R, Gaetani M, Molino E, et al. The role of bFGF on the ability of MSC to activate endogenous regenerative mechanisms in an ectopic bone formation model. Biomaterials 33, 2086-2096 (2012).

87 Kisseleva T, Brenner DA. The phenotypic fate and functional role for bone marrow-derived stem cells in liver fibrosis. J.Hepatol. (2011).

88 Liu H, Lu K, Macary PA, et al. Soluble molecules are key in maintaining the immunomodulatory activity of murine mesenchymal stromal cells. J.Cell.Sci. 125, 200-208 (2012).

89 Gehmert S, Gehmert S, Hidayat M, et al. Angiogenesis: the role of PDGF-BB on adiopse-tissue derived stem cells (ASCs). Clin. Hemorheol.Microcirc. 48, 5-13 (2011).

90 Amado LC, Saliaris AP, Schuleri KH, et al. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc.Natl.Acad.Sci.U.S.A. 102, 11474-11479 (2005).

91 Das H, George JC, Joseph M, et al. Stem cell therapy with overexpressed VEGF and PDGF genes improves cardiac function in a rat infarct model. PLoS One 4, e7325 (2009).

92 Deng Y, Zhou H, Yan C, et al. *In vitro* osteogenic induction of bone marrow stromal cells with encapsulated gene-modified bone marrow stromal cells and *in vivo* implantation for orbital bone repair. Tissue Eng.Part A. 20, 2019-2029 (2014).

93 Paul A, Nayan M, Khan AA, Shum-Tim D, Prakash S. Angiopoietin-1-expressing adipose stem cells genetically modified with baculovirus nanocomplex: investigation in rat heart with acute infarction. Int.J.Nanomedicine 7, 663-682 (2012).

94 Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Sustained expression of coagulation factor IX by modified cord blood-derived mesenchymal stromal cells. J.Gene Med. 16, 131-142 (2014).

95 Yu J, Du KT, Fang Q, et al. The use of human mesenchymal stem cells encapsulated in RGD modified alginate microspheres in the repair of myocardial infarction in the rat. Biomaterials 31, 7012-7020 (2010).

96 Park JS, Shim MS, Shim SH, et al. Chondrogenic potential of stem cells derived from amniotic fluid, adipose tissue, or bone marrow encapsulated in fibrin gels containing TGF-beta3. Biomaterials 32, 8139-8149 (2011).

97 Razavi S, Razavi MR, Kheirollahi-Kouhestani M, Mardani M, Mostafavi FS. Co-culture with neurotrophic factor secreting cells induced from adipose-derived stem cells: promotes neurogenic differentiation. Biochem.Biophys. Res.Commun. 440, 381-387 (2013).

98 Kerby A, Jones ES, Jones PM, King AJ. Co-transplantation of islets with mesenchymal stem cells in microcapsules demonstrates graft outcome can be improved in an isolated-

graft model of islet transplantation in mice. Cytotherapy 15, 192-200 (2013).

99 Bae SE, Choi DH, Han DK, Park K. Effect of temporally controlled release of dexamethasone on *in vivo* chondrogenic differentiation of mesenchymal stromal cells. J.Control.Release 143, 23-30 (2010).

100 Choi DH, Park CH, Kim IH, Chun HJ, Park K, Han DK. Fabrication of core-shell microcapsules using PLGA and alginate for dual growth factor delivery system. J.Control.Release 147, 193-201 (2010).

101 Springer ML, Hortelano G, Bouley DM, Wong J, Kraft PE, Blau HM. Induction of angiogenesis by implantation of encapsulated primary myoblasts expressing vascular endothelial growth factor. J.Gene Med. 2, 279-288 (2000).

102 Maehr R, Chen S, Snitow M, et al. Generation of pluripotent stem cells from patients with type 1 diabetes. Proc.Natl.Acad.Sci.U.S.A. 106, 15768-15773 (2009).

103 Naftanel MA, Harlan DM. Pancreatic islet transplantation. PLoS Med. 1, e58; quiz e75 (2004).

104 White SA, Shaw JA, Sutherland DE. Pancreas transplantation. Lancet 373, 1808-1817 (2009).

105 Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292, 1389-1394 (2001).

106 Godfrey KJ, Mathew B, Bulman JC, Shah O, Clement S, Gallicano GI. Stem cell-based treatments for Type 1 diabetes mellitus: bone marrow, embryonic, hepatic, pancreatic and induced pluripotent stem cells. Diabet.Med. (2011).

107 Gabr MM, Sobh MM, Zakaria MM, Refaie AF, Ghoneim MA. Transplantation of insulin-producing clusters derived from adult bone marrow stem cells to treat diabetes in rats. Exp. Clin.Transplant. 6, 236-243 (2008).

108 Ngoc PK, Phuc PV, Nhung TH, Thuy DT, Nguyet NT. Improving the efficacy of type 1 diabetes therapy by transplantation of immunoisolated insulin-producing cells. Hum. Cell 24, 86-95 (2011).

109 Wang N, Adams G, Buttery L, Falcone FH, Stolnik S. Alginate encapsulation technology supports embryonic stem cells differentiation into insulin-producing cells. J.Biotechnol. 144, 304-312 (2009).

110 Tuch BE, Hughes TC, Evans MD. Encapsulated pancreatic progenitors derived from human embryonic stem cells as a therapy for insulin-dependent diabetes. Diabetes Metab.Res.Rev. 27, 928-932 (2011).

111 Richardson T, Kumta PN, Banerjee I. Alginate encapsulation of human embryonic stem cells to enhance directed differentiation to pancreatic islet-like cells. Tissue Eng.Part A. 20, 3198-3211 (2014).

112 Collins VP, James CD. Gene and

chromosomal alterations associated with the development of human gliomas. FASEB J. 7, 926-930 (1993).

113 Shah K. Mesenchymal stem cells engineered for cancer therapy. Adv.Drug Deliv. Rev. (2011).

114 Kauer TM, Figueiredo JL, Hingtgen S, Shah K. Encapsulated therapeutic stem cells implanted in the tumor resection cavity induce cell death in gliomas. Nat.Neurosci. 15, 197-204 (2011).

115 Rege TA, Fears CY, Gladson CL. Endogenous inhibitors of angiogenesis in malignant gliomas: nature's antiangiogenic therapy. Neuro Oncol. 7, 106-121 (2005).

116 Kleinschmidt K, Klinge PM, Stopa E, et al. Alginate encapsulated human mesenchymal stem cells suppress syngeneic glioma growth in the immunocompetent rat. J.Microencapsul. 28, 621-627 (2011).

117 Goren A, Dahan N, Goren E, Baruch L, Machluf M. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. FASEB J. 24, 22-31 (2010).

118 Sayyar B, Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Cell-matrix Interactions of Factor IX (FIX)-engineered human mesenchymal stromal cells encapsulated in RGD-alginate vs. Fibrinogen-alginate microcapsules. Artif.Cells Nanomed Biotechnol. (2013).

119 Sayyar B, Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Fibronectin-Alginate microcapsules improve cell viability and protein secretion of encapsulated Factor IX-engineered human mesenchymal stromal cells. Artif.Cells Nanomed Biotechnol. (2014).

120 Lubiatowski P, Kruczynski J, Gradys A, Trzeciak T, Jaroszewski J. Articular cartilage repair by means of biodegradable scaffolds. Transplant.Proc. 38, 320-322 (2006).

121 Nejadnik H, Hui JH, Feng Choong EP, Tai BC, Lee EH. Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study. Am.J.Sports Med. 38, 1110-1116 (2010).

122 Barbero A, Grogan S, Schafer D, Heberer M, Mainil-Varlet P, Martin I. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. Osteoarthritis Cartilage 12, 476-484 (2004).

123 Munirah S, Samsudin OC, Aminuddin BS, Ruszymah BH. Expansion of human articular chondrocytes and formation of tissue-engineered cartilage: A step towards exploring a potential use of matrix-induced cell therapy. Tissue Cell (2010).

124 Buckley CT, Meyer EG, Kelly DJ. The influence of construct scale on the composition and functional properties of cartilaginous tissues engineered using bone marrow-derived mesenchymal stem cells. Tissue Eng.

Part A. 18, 382-396 (2012).

125 Pleumeekers MM, Nimeskern L, Koevoet WL, et al. The *in vitro* and *in vivo* capacity of culture-expanded human cells from several sources encapsulated in alginate to form cartilage. Eur.Cell.Mater. 27, 264-80; discussion 278-80 (2014).

126 Perrier E, Ronziere MC, Bareille R, Pinzano A, Mallein-Gerin F, Freyria AM. Analysis of collagen expression during chondrogenic induction of human bone marrow mesenchymal stem cells. Biotechnol.Lett. 33, 2091-2101 (2011).

127 Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp.Cell Res. 238, 265-272 (1998).

128 Tuli R, Tuli S, Nandi S, et al. Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. J.Biol.Chem. 278, 41227-41236 (2003).

129 Mello MA, Tuan RS. Effects of TGF-beta1 and triiodothyronine on cartilage maturation: *in vitro* analysis using long-term high-density micromass cultures of chick embryonic limb mesenchymal cells. J.Orthop.Res. 24, 2095-2105 (2006).

130 Patil AS, Sable RB, Kothari RM. An update on transforming growth factor-beta (TGF-beta): sources, types, functions and clinical applicability for cartilage/bone healing. J.Cell. Physiol. 226, 3094-3103 (2011).

131 Solorio LD, Vieregge EL, Dhami CD, Dang PN, Alsberg E. Engineered cartilage via self-assembled hMSC sheets with incorporated biodegradable gelatin microspheres releasing transforming growth factor-beta1. J.Control. Release (2011).

132 Dashtdar H, Rothan HA, Tay T, et al. A preliminary study comparing the use of allogenic chondrogenic pre-differentiated and undifferentiated mesenchymal stem cells for the repair of full thickness articular cartilage defects in rabbits. J.Orthop.Res. 29, 1336-1342 (2011).

133 Diduch DR, Jordan LC, Mierisch CM, Balian G. Marrow stromal cells embedded in alginate for repair of osteochondral defects. Arthroscopy 16, 571-577 (2000).

134 Valiani A, Hashemibeni B, Esfandiary E, Ansar MM, Kazemi M, Esmaeili N. Study of carbon nano-tubes effects on the chondrogenesis of human adipose derived stem cells in alginate scaffold. Int.J.Prev.Med. 5, 825-834 (2014).

135 Pili D, Tranquilli Leali P. Biomaterials and bone. Aging Clin.Exp.Res. 23, 74-75 (2011).

136 Bouhadir KH, Lee KY, Alsberg E, Damm KL, Anderson KW, Mooney DJ. Degradation of partially oxidized alginate and its potential application for tissue engineering. Biotechnol. Prog. 17, 945-950 (2001).

137 Bhat A, Hoch AI, Decaris ML, Leach JK.

Alginate hydrogels containing cell-interactive beads for bone formation. FASEB J. 27, 4844-4852 (2013).

138 Chen W, Zhou H, Tang M, Weir MD, Bao C, Xu HH. Gas-Foaming Calcium Phosphate Cement Scaffold Encapsulating Human Umbilical Cord Stem Cells. Tissue Eng.Part A. (2011).

139 Zhao L, Weir MD, Xu HH. An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. Biomaterials 31, 6502-6510 (2010).

140 Zhou H, Xu HH. The fast release of stem cells from alginate-fibrin microbeads in injectable scaffolds for bone tissue engineering. Biomaterials 32, 7503-7513 (2011).

141 Huang S, Jia S, Liu G, Fang D, Zhang D. Osteogenic differentiation of muscle satellite cells induced by platelet-rich plasma encapsulated in three-dimensional alginate scaffold. Oral Surg.Oral Med.Oral Pathol.Oral Radiol.Endod. (2012).

142 Burridge PW, Keller G, Gold JD, Wu JC. Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. Cell.Stem Cell. 10, 16-28 (2012).

143 Cortes-Morichetti M, Frati G, Schussler O, et al. Association between a cell-seeded collagen matrix and cellular cardiomyoplasty for myocardial support and regeneration. Tissue Eng. 13, 2681-2687 (2007).

144 Christman KL, Vardanian AJ, Fang Q, Sievers RE, Fok HH, Lee RJ. Injectable fibrin scaffold improves cell transplant survival, reduces infarct expansion, and induces neovasculature formation in ischemic myocardium. J.Am.Coll. Cardiol. 44, 654-660 (2004).

145 Kofidis T, Lebl DR, Martinez EC, Hoyt G, Tanaka M, Robbins RC. Novel injectable bioartificial tissue facilitates targeted, less invasive, large-scale tissue restoration on the beating heart after myocardial injury. Circulation 112, I173-7 (2005).

146 Al Kindi AH, Asenjo JF, Ge Y, et al. Microencapsulation to reduce mechanical loss of microspheres: implications in myocardial cell therapy. Eur.J.Cardiothorac.Surg. 39, 241-247 (2011).

147 Tang J, Xie Q, Pan G, Wang J, Wang M. Mesenchymal stem cells participate in angiogenesis and improve heart function in rat model of myocardial ischemia with reperfusion. Eur.J.Cardiothorac.Surg. 30, 353-361 (2006).

148 Xiang MX, He AN, Wang JA, Gui C. Protective paracrine effect of mesenchymal stem cells on cardiomyocytes. J.Zhejiang Univ.Sci.B. 10, 619-624 (2009).

149 Nayan M, Paul A, Chen G, Chiu RC, Prakash S, Shum-Tim D. Superior therapeutic potential of young bone marrow mesenchymal stem cells by direct intramyocardial delivery in aged recipients with acute myocardial infarction: *in vitro* and *in vivo* investigation. J.Tissue Eng. 2011, 741213 (2011).

150 Fu Y, Azene N, Ehtiati T, et al. Fused X-ray and MR imaging guidance of intrapericardial delivery of microencapsulated human mesenchymal stem cells in immunocompetent swine. Radiology 272, 427-437 (2014).

151 Wright EJ, Farrell KA, Malik N, et al. Encapsulated glucagon-like peptide-1-producing mesenchymal stem cells have a beneficial effect on failing pig hearts. Stem Cells Transl. Med. 1, 759-769 (2012).

152 Jing D, Parikh A, Tzanakakis ES. Cardiac cell generation from encapsulated embryonic stem cells in static and scalable culture systems. Cell Transplant. 19, 1397-1412 (2010).

153 Dalton PD, Mey J. Neural interactions with materials. Front.Biosci. 14, 769-795 (2009).

154 Prang P, Muller R, Eljaouhari A, et al. The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. Biomaterials 27, 3560-3569 (2006).

155 Horner PJ, Gage FH. Regenerating the damaged central nervous system. Nature 407, 963-970 (2000).

156 Li X, Liu T, Song K, et al. Culture of neural stem cells in calcium alginate beads. Biotechnol.Prog. 22, 1683-1689 (2006).

157 Cunha C, Panseri S, Villa O, Silva D, Gelain F. 3D culture of adult mouse neural stem cells within functionalized self-assembling peptide scaffolds. Int.J.Nanomedicine 6, 943-955 (2011).

158 Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY. Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat.Biotechnol. 20, 1103-1110 (2002).

159 Matyash M, Despang F, Mandal R, Fiore D, Gelinsky M, Ikonomidou C. Novel soft alginate hydrogel strongly supports neurite growth and protects neurons against oxidative stress. Tissue Eng.Part A. 18, 55-66 (2012).

160 Ashton RS, Banerjee A, Punyani S, Schaffer DV, Kane RS. Scaffolds based on degradable alginate hydrogels and poly(lactide-co-glycolide) microspheres for stem cell culture. Biomaterials 28, 5518-5525 (2007).

161 Sidhu K, Kim J, Chayosumrit M, Dean S, Sachdev P. Alginate microcapsule as a 3D platform for propagation and differentiation of human embryonic stem cells (hESC) to different lineages. J.Vis.Exp. (61). pii: 3608. doi, 10.3791/3608 (2012).

162 Heile AM, Wallrapp C, Klinge PM, et al. Cerebral transplantation of encapsulated mesenchymal stem cells improves cellular pathology after experimental traumatic brain injury. Neurosci.Lett. 463, 176-181 (2009).

163 Klinge PM, Harmening K, Miller MC, et al. Encapsulated native and glucagon-like peptide-1 transfected human mesenchymal stem cells in a transgenic mouse model of Alzheimer's disease. Neurosci.Lett. 497, 6-10 (2011).

164 Heile A, Brinker T. Clinical translation of stem cell therapy in traumatic brain injury: the potential of encapsulated mesenchymal cell biodelivery of glucagon-like peptide-1. Dialogues Clin.Neurosci. 13, 279-286 (2011).

165 Dvir-Ginzberg M, Elkayam T, Cohen S. Induced differentiation and maturation of newborn liver cells into functional hepatic tissue in macroporous alginate scaffolds. FASEB J. 22, 1440-1449 (2008).

166 Glicklis R, Shapiro L, Agbaria R, Merchuk JC, Cohen S. Hepatocyte behavior within three-dimensional porous alginate scaffolds. Biotechnol.Bioeng. 67, 344-353 (2000).

167 Khalil M, Shariat-Panahi A, Tootle R, et al. Human hepatocyte cell lines proliferating as cohesive spheroid colonies in alginate markedly upregulate both synthetic and detoxificatory liver function. J.Hepatol. 34, 68-77 (2001).

168 Hamazaki T, Iiboshi Y, Oka M, et al. Hepatic maturation in differentiating embryonic stem cells *in vitro*. FEBS Lett. 497, 15-19 (2001).

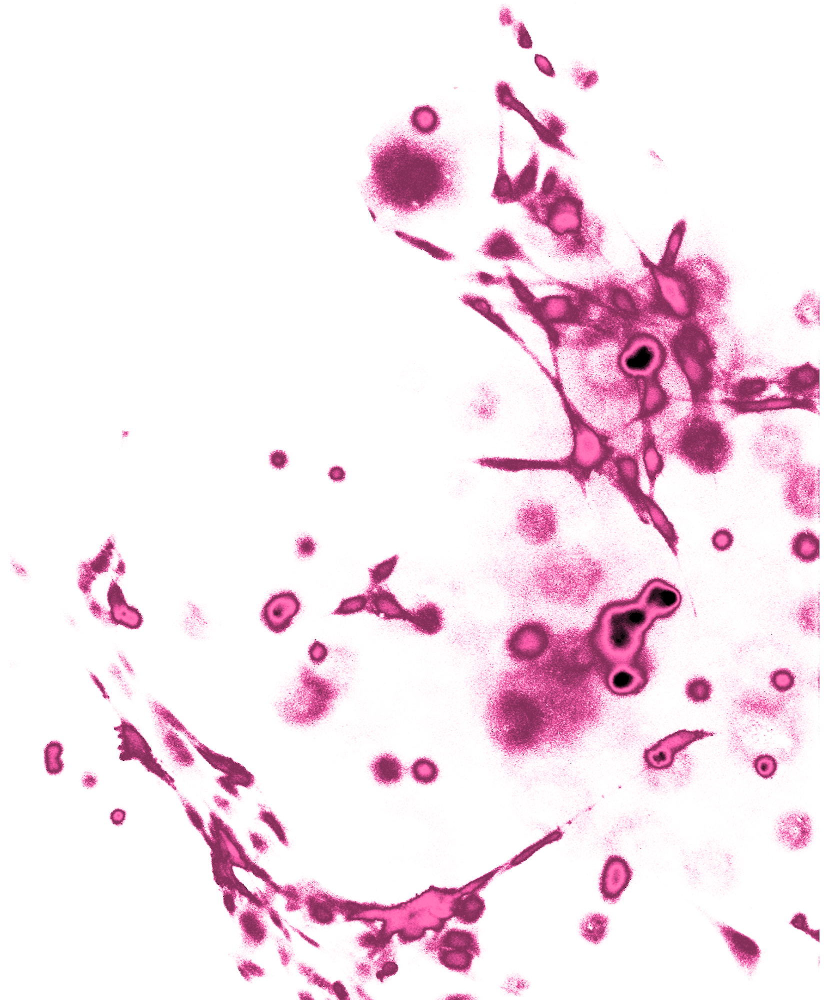
169 Zhou M, Li P, Tan L, Qu S, Ying QL, Song H. Differentiation of mouse embryonic stem cells into hepatocytes induced by a combination of cytokines and sodium butyrate. J.Cell.Biochem. 109, 606-614 (2010).

170 Maguire T, Novik E, Schloss R, Yarmush M. Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes. Biotechnol.Bioeng. 93, 581-591 (2006).

171 Liu ZC, Chang TM. Coencapsulation of hepatocytes and bone marrow stem cells: *in vitro* conversion of ammonia and *in vivo* lowering of bilirubin in hyperbilirubemia Gunn rats. Int.J.Artif.Organs 26, 491-497 (2003).

172 Trouche E, Girod Fullana S, Mias C, et al. Evaluation of alginate microspheres for mesenchymal stem cell engraftment on solid organ. Cell Transplant. 19, 1623-1633 (2010).

Objectives



Cell microencapsulation is a promising therapeutic strategy which involves the immobilization of cells into a polymeric matrix usually surrounded by a semipermeable membrane. To date, a wide variety of cells have been encapsulated in alginate microcapsules with the aim of achieving a sustained delivery of numerous therapeutic factors.

One of the main limitations of this therapy is the maintenance of high cell viability, as cell apoptosis and necrosis within the capsules impairs the delivery of therapeutic factors. The modification of polymers with extracellular matrix (ECM) proteins or its derivatives is one of exciting strategy to address this issue. The short-sequence peptide derived from fibronectin arginine-glycine-aspartate (RGD) has been widely employed to promote and regulate cellular interactions, providing the encapsulated cells with a microenvironment that more closely mimics their natural conditions. However, some discrepancies regarding the therapeutic benefits of RGD collected in recent works have led to questioning its effect, increasing the need to assess this adhesion ligand exhaustively.

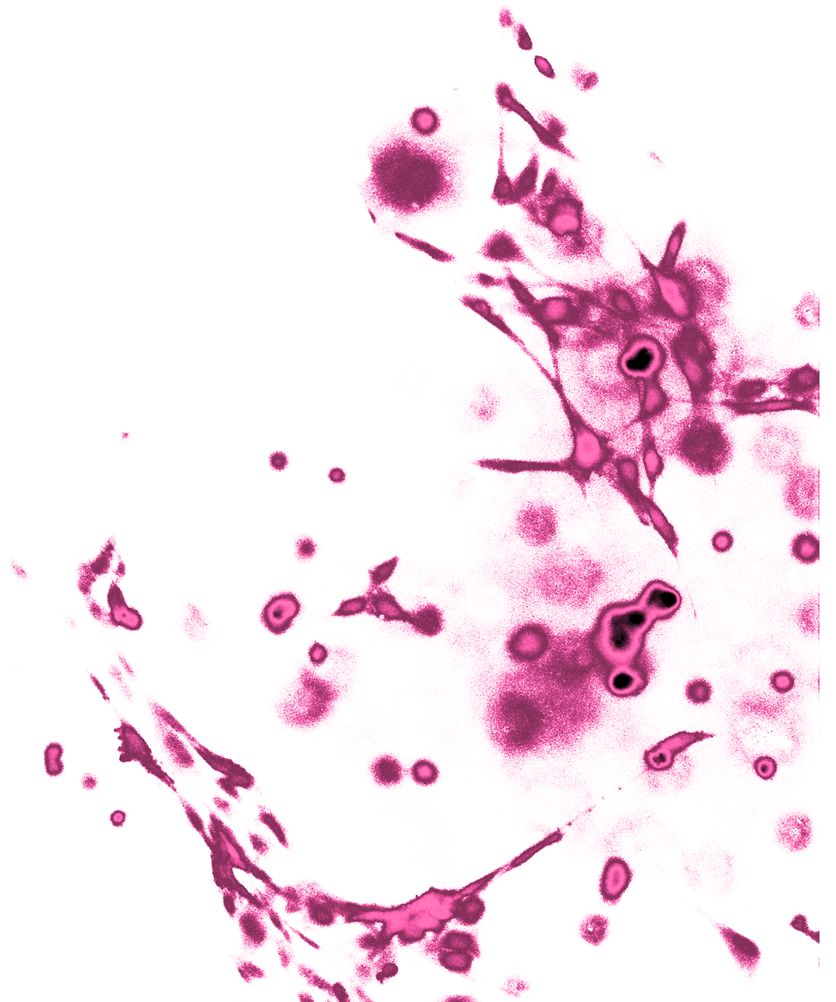
Thus, the specific goals of this work are the following:

- To design alginate microcapsules elaborated with different RGD densities to assess the behavior of immobilized cells in terms of viability, proliferation and therapeutic factor secretion.

- To evaluate the influence of different RGD densities on the behavior of diverse cell types in order to determine the optimal adhesion ligand density that provides the highest cell activity for each cell type.

- To compare the effects of different RGD densities *in vitro* with the results obtained *in vivo*.

Experimental design



Chapter 1

Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems

Journal of Drug Targeting 27, 1-7 (2015)

Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems

Ane Garate[1,2,a], Edorta Santos[1,2,a], José Luis Pedraz[1,2], Rosa María Hernández[1,2], Gorka Orive[1,2]

1NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Vitoria, Spain.
2Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria, Spain.
aThese two authors contributed equally to this work.

ABSTRACT

Background: The inclusion of the tripeptide Arg-Gly-Asp (RGD) in otherwise inert biomaterials employed for cell encapsulation has been observed to be an effective strategy to provide the immobilized cells with a more suitable microenvironment.
Purpose: The objective of the present study was to determine the impact of different RGD densities on the behavior of baby hamster kidney (BHK) fibroblasts able to secrete vascular endothelial growth factor (VEGF) encapsulated in alginate microcapsules.
Methods: Alginate was modified by varying the concentration of RGD peptides in the coupling reaction. After obtaining four different types of alginate, cells were encapsulated in alginate-poly-l-lysine-alginate (APA) microcapsules.
Results and discussion: The results obtained after viability, cell proliferation and VEGF secretion assays showed that the inclusion of RGD in alginate enhances the functionality of immobilized cells, obtaining the highest values with the high-intermediate RGD density.
Conclusion: These results put in evidence that alginate modification influences the behavior of immobilized cells but even more that the employed density of the tripeptide is of crucial importance, obtaining in some cases even excessive activity of the encapsulated cells.

Keywords
RGD density, microcapsule, alginate, BHK fibroblasts

1. Introduction

In the last decades, encapsulation of genetically modified cells has been employed as a promising therapy for "de novo" delivery of diverse therapeutic factors to treat many diseases or disorders [1-4]. Thus, a broad range of cell lines has been enclosed within polymeric matrices surrounded by a semipermeable membrane designed to immune-protect the cell content from both mechanical stress and host's cellular immune rejection, allowing a safe transplantation [5-9]. Although the effectiveness of this therapy depends on many factors, the key to its success is to preserve a good cell viability in order to achieve the last goal of this technology, a controlled and sustained delivery of the therapeutic agent. For that reason, increasingly sophisticated three-dimensional (3D) scaffolds are designed mimicking the natural physiological environment of immobilized cells, enhancing their survival and well-being within the matrices [10-13].

Alginate is a natural biomaterial widely used in cell encapsulation due to its several favorable properties, but it does not provide sufficient cues for cell-matrix interactions, being an inert polymer [14,15]. One way to prolong cell survival in these non-cell-interactive matrices is to modify them with the amino acid sequence Arginine-Glicine-Aspartate (RGD), a short adhesion peptide found in fibronectin and other natural components of the ECM. The use of this tripeptide offers some advantages over the use of the whole protein; for example, simplicity, cost effectiveness, easy manipulation for functionalization and low immune response [16,17]. For these reasons, RGD has generated attention as a potential means to provide inert polymers with biological cues and thus extending the long term viability of the immobilized cells.

Although there is a significant biological foundation to support the activity of RGD moiety and an exhaustive literature has established that it is highly effective at promoting the attachment of numerous cell types in diverse biomaterials [18-22], some inconsistencies collected in recent works, especially *in vivo*, have led to questioning its actual effectiveness. Indeed, the variability that offers this adhesion ligand might become a disadvantage in certain cases, which has generated some discrepancies in the scientific community to date [23-

27]. Among others, the use of different RGD ligand types, the differences in RGD presentation patterns or the effect of the microenvironment enhancing/ decreasing its activity are variables that must be taken into account when using RGD [28-32]. In this regard, the influence of adhesion ligand density is one of the main parameters that must be defined for each particular cell and application, being pivotal to obtain the optimum functionality of immobilized cells [26,33,34].

In the present study, vascular endothelial growth factor (VEGF) secreting baby hamster kidney (BHK) fibroblasts were chosen as a cell model. With the aim to evaluate the behavior and the secretion ability of this cell type in RGD-coupled microcapsules, different densities of the adhesion ligand (DS 1, DS 5, and DS 10) were incorporated in alginate matrices to further analyze the number of viable cells per capsule, cell proliferation and morphology and, most importantly, the secretion of the therapeutic factor.

2. Materials and methods

2.1. Cell culture

Baby hamster kidney (BHK) fibroblasts genetically engineered to produce human VEGF, were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine to a final concentration of 2 mM, 4.5 g/L glucose and 1% antibiotic/antimycotic solution. Cells were maintained in culture at 37 ºC in a humidified 5% CO_2/95% air atmosphere and passaged every 2-3 days. All reagents were purchased from Life technologies, Spain.

2.2. Incorporation of adhesion molecules into alginate

Alginate was chemically modified by the aqueous carbodiimide chemistry. A water soluble carbodiimide, (1-ethyl-dimethylaminopropyl) carbodiimide (EDC), was used to form amide linkages between amine containing molecules and the carboxylate moieties on the alginate polymer backbone, with a reaction efficiency of approximately 80% [35]. The total number of RGD peptides per alginate chain, defined as the degree of substitution (DS) [36], was modified by varying the concentration of RGD peptides in the coupling reaction, obtaining four different types of alginate:

DS 0 (No modified alginate), DS 1 (0.112 mM), DS 5 (0.5 mM) and DS 10 (1.12 mM).

2.3. Cell microencapsulation

BHK fibroblasts genetically modified to release VEGF were incorporated into 3D alginate-poly-l-lysine-alginate (APA) microcapsules using an electrostatic droplet generator with brief modifications of the procedure designed by Lim and Sun [37]. Briefly, cells were harvested from monolayer cultures using trypsin- EDTA (Life technologies), filtered through a 40 µm pore mesh and suspended in four different solutions of 1.5% (w/v) sodium alginate (DS 0, DS 1, DS 5, DS 10) at $5x10^6$ cells/ml density. The resulted suspensions were extruded in a sterile syringe through a 0.35 mm needle at a 5.9 mL/h flow rate using a peristaltic pump. The resulting alginate particles were collected in a 55mM $CaCl_2$ solution and maintained under agitation for 15 min after the end of the process to ensure complete gelation of all the beads. Then, the obtained particles were suspended in 0.05% PLL solution for 5 min, washed twice with 10 mL of manitol 1% and coated again with another layer of 0.1% alginate for 5 min. All the process was carried out under aseptic conditions at room temperature, and resulting microcapsules were cultured in complete medium at 37 ºC in a 5% CO_2/95% air atmosphere standard incubator. Ultra pure low-viscosity high guluronic acid alginate (UPLVG) was purchased from FMC Biopolymer, Norway, and poly-l-lysine (PLL hidrobromide Mw 15 000–30 000 Da) was obtained from Sigma-Aldrich (St. Louis,MO, USA).

2.4. Cell Viability

Cells entrapped into APA microcpasules were dyed with the LIVE/DEAD kit (Life technologies) following manufacturer's indications. After 30 min, fluorescence micrographs were taken using an epi-fluorescence microscope (Nikon TSM).

2.5. Quantification of the total number of living cells per capsule

In order to determine the exact number of living cells quantitatively, enclosed cells were firstly de-encapsulated with 500 µg/mL of alginate lyase (Sigma-Aldrich). LIVE/DEAD kit (Life technologies) was used to differentiate living and dead cells. After incubation of samples for 20 min at room temperature and protected from light, cells were counted by means of flow cytometry (BD FACSCalibur) using Trucount Tubes (BD). All samples were assayed in triplicate

for all groups, and obtained values are shown as mean of 3 independent samples ± S.D per study group.

2.6. Measurement of VEGF secretion

Encapsulated BHK fibroblasts supernatants were assayed for VEGF secretion using the Human VEGF Quantikine ELISA Kit purchased from R&D Systems (Minneapolis, MN). Standards and samples were run in duplicate according to the procedure specified in the kit. The VEGF secretion of the equivalent of 1.67×10^5 cells/mL was measured for a 24 h release period in triplicate per study group, and results are expressed as mean ± S.D.

2.7. Cell proliferation assay

The equivalent of 2×10^4 cells/100 µL (≈100 microcapsule/ well) was placed into each well of 96-well plate. All groups were incubated with complete medium supplemented with 10% FBS except the negative control group, which was incubated with starving medium supplemented with 0.1% FBS. After 24 h, the encapsulated cells were incubated in the presence of 10 µM BrdU for an additional day, except non-specific binding control group. The third day cells were de-encapsulated using 500 µg/mL of alginate lyase (Sigma-Aldrich) and assayed for BrdU uptake using Cell Proliferation Biotrak ELISA System (Amersham, NJ, USA) following manufacturer's indications. Absorbance measurements of the non-specific binding control group (without BrdU) were subtracted from the rest of the groups, and results were normalized with the corresponding negative control (microcapsules incubated with 0.1 % FBS) for each experiment. Data are shown as mean of 5 independent samples ± S.D per study group.

2.8. Cell morphology. Determination of F actin

Microcapsules (100 µl of capsules) were fixed using 4 % paraformaldehyde, washed in pre-warmed DPBS and permeabilized by 0.1 % Triton X-100 for 10 minutes. The cytoskeleton of encapsulated cells was stained with Alexa Fluor 488 phalloidin (Life technologies). A volume of 15 µL methanolic stock solution was mixed for 30 min in the dark in 200 µl DPBS containing 1 % bovine serum albumin to reduce nonspecific background. The nucleus of the cells were dyed with Hoechst (1 µg/ml) and the samples were analyzed by inverted confocal microscopy (Olympus

Fluoview 500 Confocal Microscopy).

2.9. Statistical analysis

Data are presented as mean ± S.D. One-way ANOVA and post-hoc test were used in multiple comparisons. The Bonferroni or Tamhane post-hoc test was applied according to the result of the Levene test of homogeneity of variances. In the case of non-normally distributed data, Mann-Whitney non-parametric analysis was used. All statistical computations were performed using SPSS 20 (SPSS, Inc., Chicago, IL).

3. Results and discussion

3.1. Microcapsules characterization and cell viability evaluation

VEGF secreting BHK fibroblasts were encapsulated in 4 different alginate matrices modified with increasing RGD densities (DS 0, DS 1, DS 5 and DS 10) in order to test the behavior of this cell line during a month (Fig. 1). All types of microcapsules had a spherical morphology without irregularities in the surface and an homogeneous size distribution (diameter 450-470 µm). Moreover, the number of cells in each capsule was similar in all groups at the beginning of the study

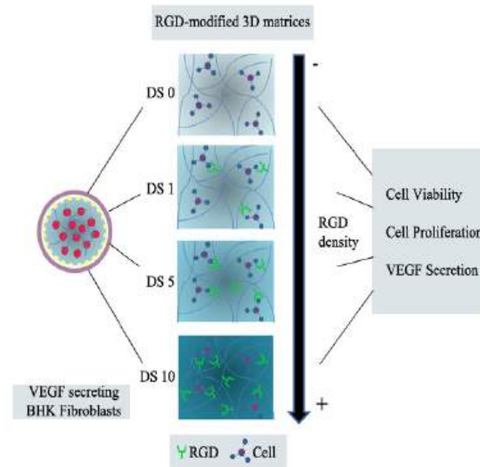


Figure 1. Schematic illustration of VEGF-secreting BHK fibroblasts encapsulated in 4 alginate matrices elaborated with different RGD densities.

(≈200 cells/cap) (Fig. 2 A-D).

To further evaluate the potential impact of RGD bioactivated matrices on BHK fibroblasts, firstly we measured the viability of immobilized cells. Green fluorescence of micrographs taken by day 15 showed a similar number of living cells in all studied microcapsules, without visual differences between them (Fig. 2 E-H). However, after 30 days of encapsulation we could clearly observe the trend of fibroblasts to form living cell aggregates in the presence of RGD (Fig. 2 I-L), while cells entrapped in no modified microcapsules remained with

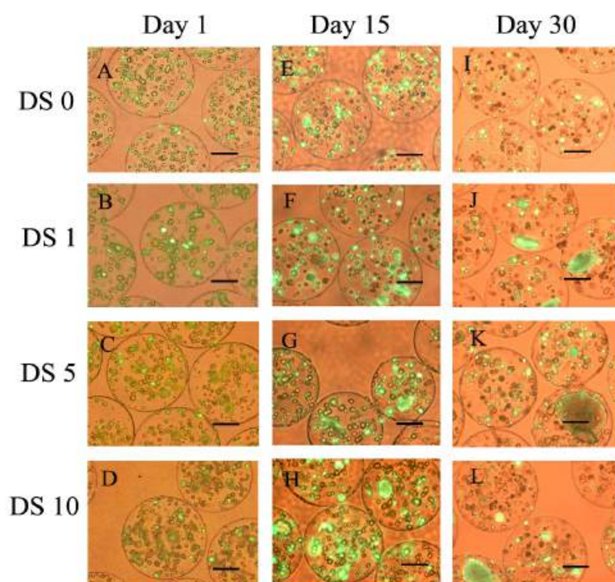


Figure 2. Fluorescence micrographs taken by day 1 (A-D), day 15 (E-H) and day 30 (I-L). Scale bars = 100 μm.

the same distribution observed at day 1. This finding come along with other previously reported results in the literature revealing that this cell line tends to form cell aggregates in the presence of adhesion ligands [38,39].

In order to provide more accurate data about the viability of encapsulated fibroblasts, the number of living cells was measured by flow cytometry. These results suggested that although by day 15 there were not statistically significant differences between all groups (values around 50 % in all cases) (Fig. 3A), at day 30 the RGD-enriched microcapsules maintained higher viability rates than unmodified ones (24.62 % ± 3.9), being DS 5 the group that exhibited the highest normalized values (286.42 % ± 26.62) ($p<0.001$)(Fig. 3B). This data confirmed that the incorporation of RGD in otherwise inert biomaterials plays a significant role on the survival of immobilized cells, probably due to the interaction between the cells and the biomaterial which additionally leads to the stabilization of the cell-polymer biosystem [20,40].

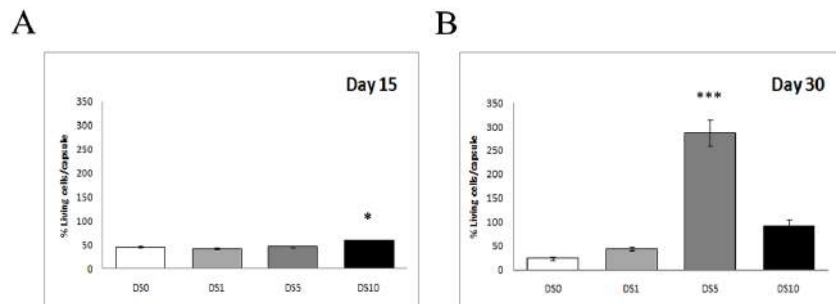


Figure 3. Percentage of living cells after 15 (A) and 30 days (B) of encapsulation. The number of living cells obtained for the day 0 was considered as 100% in each microcapsules group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). Statistical significance *p < 0.05 and ***p < 0.001 compared with DS 0 group.

3.2. Proliferation and aggregates formation in RGD-coupled matrices

The results of BrdU uptake by day 15 revealed a higher proliferation activity in all RGD-coupled alginate groups, being the highest one the DS 5 group with a 3.22 ± 0.50-fold higher BrdU uptake with respect to DS 0 group (p<0.001). The differences were lower when this group was compared to DS 1 (p<0.01) or DS 10 groups (Fig. 4A). It has been previously described that fibroblasts have a basal proliferation activity when entrapped within 3D scaffolds [41]. However, according with obtained results, when they are embedded in modified alginate the proliferation of these cells increases considerably leading in some cases to cell aggregates as observed in the figure 2.

Although the rest of groups maintained a similar BrdU uptake by day 30, DS 5 group continued with a higher proliferation activity achieving values of 2.42 ± 0.1-fold higher with respect to DS 0 group (p<0.001) (Fig. 4B). It must be taken into account that such uncontrolled proliferation may result in a rupture of the microcapsule, with the subsequent spread out of cells compromising the stability of the implant and their survival. To avoid this event, diverse alternatives should be analized, such as increasing the mechanical stability of the microcapsules, reducing the initial encapsulated cell density, the inclusion of biosafety genes to inactivate encapsulated cells and control their therapeutic effects or the choice of the optimum adhesion ligand

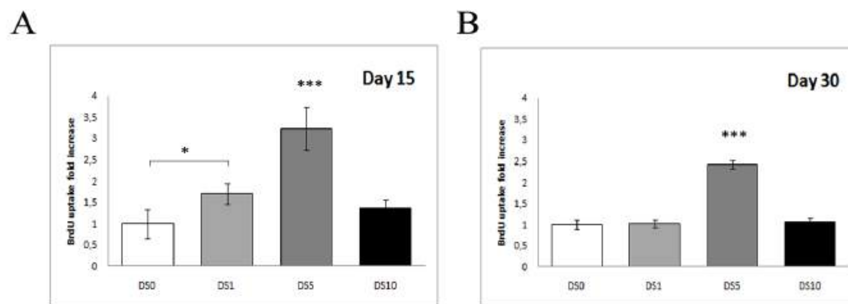


Figure 4. BrdU uptake after 15 (A) and 30 days (B) of encapsulation. The results were normalized with those obtained with DS 0 group each day. Bar graphs symbolize the mean ± S.D (n=5). Statistical significance *p < 0.05 and ***p < 0.001 compared with DS 0 group.

(RGD density).

3.3. Therapeutic factor secretion

The results of VEGF secretion from BHK fibroblasts proved that cells enclosed within microcapsules elaborated with RGD-coupled alginate were able to secrete more quantity of therapeutic factor comparing with cells within no modified alginate scaffolds. Although there were not significant differences in both analyzed days (Fig. 5), DS 5 was the group with the highest secretion of VEGF the last day of the study achieving values over 1200 % ± 59 (Fig. 5B), 6-fold higher quantity than VEGF secreted by cells encapsulated in no modified alginate. However, there is a clear trend in the results, which in accordance with cell viability assays, suggests that DS 5 is the RGD density that induces the highest secretion ability in this particular cell type. Most likely, this is directly related to the superior proliferation observed in this group.

Several studies carried out in the field were intended to enhance the viability of encapsulated cells and therefore to achieve an increase on the release of therapeutic factor secreted by cells. With that purpose different molecules derived from ECM have been employed to modify polymers such as alginate [11,42,43]. As demonstrated in this study, the inclusion of small peptides such as RGD might be a potential strategy to achieve that aim. Results show that maintaining the same number of encapsulated cells, the production of therapeutic factor can be increased. However, it is important

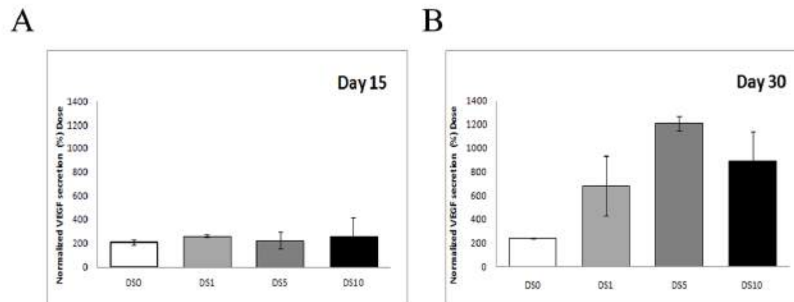


Figure 5. VEGF secretion after 15 (A) and 30 days (B) of encapsulation. Therapeutic factor secretion levels obtained for the day 0 were considered as 100% in each microcapsule group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3).

to notice that the enhancement of that production by the inclusion of this type of moiety may occur in an uncontrolled way, and in some cases this fact could result in appearance of side effects [44]. In this sense, the secretion obtained over a month by the DS 5 group may be excessive, and an exhaustive *in vivo* evaluation is necessary to estimate the dose required to achieve the best results.

Extensive research has been carried out regarding the formation of multicellular structures and the direct relation between the high proliferation activity of fibroblasts and the increase of their therapeutic factor production ability [38,39]. Our data come along with the previous ones which suggest that aggregation of cells may lead to improve cell function, such as the increase of therapeutic factor secretion. Altogether, these results suggest that for the particular case of fibroblasts, DS 5 was the peptide density with more capacity to promote the highest cell viability, proliferation activity and secretion of VEGF. Nevertheless, in a recent work published by our research group, we could observe that in the case of C_2C_{12} myoblasts, DS 1 was the group which obtained the highest activity *in vitro* [33]. It is known that RGD acts in a cell dependent way and these results also put forward the same observation [27,39]. In addition, the results collected here come along with previous studies reporting that intermediate levels of the triamino-acid sequence are optimal to obtain the maximum functionality of enclosed cells while the incorporation of excessively high densities of RGD may be inhibitory

[45,46]. This could be explained by the fact that strong adhesions resulting from too much bound receptors may impede cell division, producing an inhibitory effect when the employed densities of RGD are too high.

3.4. Change of cell morphology: F-actin

The interaction of fibroblasts with 3D matrices was evaluated by confocal microscopy. The staining of F-actin filaments with phalloidin Alexa Fluor 488 permits the observation of some filopodia-like membrane extensions when cells were immobilized in RGD-coupled alginates (Fig. 6). This filament alignment pattern was not observed in unmodified alginate microcapsules, where BHK fibroblasts retained the typical round shape with no detectable cytoplasm extensions. Although the cytoplasm extensions collected in the pictures are not very clear, unlike in DS 0 group, some surface modifications may be observed in alginate-modified groups. These cell surface modifications indicate that fibroblasts embedded in the presence of cross-linked RGD peptides have the ability to interact with the biomimetic cues provided by this moiety. Upon ligand binding, integrins can trigger downward

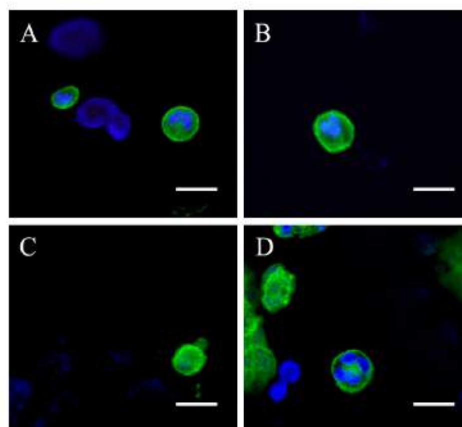


Figure 6. Cytoskeleton organizations of fibroblasts encapsulated in microcapsules elaborated with four different types of alginate DS 0 (A), DS 1 (B), DS 5 (C) and DS 10 (D). The cells inside APA microcapsules were stained with phalloidin Alexa Fluor 488 for F-actin (green) and Hoechst (blue) for nucleus. Scale bars = 20 µm.

biochemical signals inside the cells inducing multiple responses such as cell survival or proliferation [47-49].

Although we concluded that DS 5 is the optimal ligand density to obtain the highest activity on BHK fibroblasts, the lack of an *in vivo* study to observe the effects of different VEGF doses and excessive proliferation activity showed *in vitro* presents a limitation of the present work. Moreover, the high variability obtained in the samples of VEGF secretion

assay hindered the statistical analysis not getting statistical differences in the provided data. However, we could provide interesting results about the importance of the RGD ligand density achieving maximum activity of enclosed cells with intermediate levels of the sequence although depending on the intended application other scaffold designs can be preferable to avoid the high functionality obtained by the group DS 5 on immobilized cells. Future studies are required to more precisely evaluate the biological mechanisms behind RGD-cell interactions that regulate cell behavior and therapeutic factor secretion.

4. Conclusions

In summary, these preliminary results suggest that RGD density is an important factor to take into account in the design of drug delivery biosystems. Moreover, the role of RGD over cell behavior should be evaluated individually and carefully for each cell line, since its effects may be different depending on the cell type. A judicious choice of ligand density is critical for the efficacy of this therapeutic strategy and tailoring the properties of the scaffold to every cell will result of paramount importance to obtain improved and more controllable results with increased biosafety.

Acknowledgements

Authors would like to thank Ricardo Andrade for technical help with confocal microscopy. The authors also gratefully acknowledge the support to research cell microencapsulation from the “Ministerio de Ciencia e Innovación”, University of the Basque Country UPV/EHU (UFI11/32) and FEDER funds (SAF2008-03157). A. Garate thanks the “Gobierno Vasco (Departamento de Educación, Universidades e Investigación)” for the Ph.D fellowship.

References

1 Acarregui A, Murua A, Pedraz JL, Orive G, Hernandez RM. A perspective on bioactive cell microencapsulation. BioDrugs 26, 283-301 (2012).

2 Calafiore R, Basta G. Clinical application of microencapsulated islets: Actual prospectives on progress and challenges. Adv.Drug Deliv. Rev. (2013).

3 Santos E, Pedraz JL, Hernandez RM, Orive G. Therapeutic cell encapsulation: ten steps

towards clinical translation. J.Control.Release 170, 1-14 (2013).

4 Orive G, Santos E, Pedraz JL, Hernandez RM. Application of cell encapsulation for controlled delivery of biological therapeutics. Adv.Drug Deliv.Rev. (2013).

5 Murua A, de Castro M, Orive G, Hernandez RM, Pedraz JL. *In vitro* characterization and *in vivo* functionality of erythropoietin-secreting cells immobilized in alginate-poly-L-lysine-alginate microcapsules. Biomacromolecules 8, 3302-3307 (2007).

6 Ngoc PK, Phuc PV, Nhung TH, Thuy DT, Nguyet NT. Improving the efficacy of type 1 diabetes therapy by transplantation of immunoisolated insulin-producing cells. Hum.Cell 24, 86-95 (2011).

7 Luca G, Fallarino F, Calvitti M, et al. Xenograft of microencapsulated sertoli cells reverses T1DM in NOD mice by inducing neogenesis of beta-cells. Transplantation 90, 1352-1357 (2010).

8 Klinge PM, Harmening K, Miller MC, et al. Encapsulated native and glucagon-like peptide-1 transfected human mesenchymal stem cells in a transgenic mouse model of Alzheimer's disease. Neurosci.Lett. 497, 6-10 (2011).

9 Grandoso L, Ponce S, Manuel I, et al. Long-term survival of encapsulated GDNF secreting cells implanted within the striatum of parkinsonized rats. Int.J.Pharm. 343, 69-78 (2007).

10 Markusen JF, Mason C, Hull DA, et al. Behavior of adult human mesenchymal stem cells entrapped in alginate-GRGDY beads. Tissue Eng. 12, 821-830 (2006).

11 Orive G, De Castro M, Kong HJ, et al. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. J.Control.Release 135, 203-210 (2009).

12 Shin H, Jo S, Mikos AG. Biomimetic materials for tissue engineering. Biomaterials 24, 4353-4364 (2003).

13 Stupack DG, Cheresh DA. Get a ligand, get a life: integrins, signaling and cell survival. J.Cell. Sci. 115, 3729-3738 (2002).

14 Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog.Polym.Sci. 37, 106-126 (2012).

15 Augst AD, Kong HJ, Mooney DJ. Alginate hydrogels as biomaterials. Macromol.Biosci. 6, 623-633 (2006).

16 Collier JH, Segura T. Evolving the use of peptides as components of biomaterials. Biomaterials 32, 4198-4204 (2011).

17 Williams DF. The role of short synthetic adhesion peptides in regenerative medicine; the debate. Biomaterials 32, 4195-4197 (2011).

18 Wang X, Ye K, Li Z, Yan C, Ding J. Adhesion, proliferation, and differentiation of mesenchymal stem cells on RGD nanopatterns of varied nanospacings. Organogenesis 9,

(2013).

19 Xiang M, Lin Y, He G, et al. Correlation between biological activity and binding energy in systems of integrin with cyclic RGD-containing binders: a QM/MM molecular dynamics study. J.Mol.Model. 18, 4917-4927 (2012).

20 Ruoslahti E. RGD and other recognition sequences for integrins. Annu.Rev.Cell Dev. Biol. 12, 697-715 (1996).

21 Benoit YD, Groulx JF, Gagne D, Beaulieu JF. RGD-Dependent Epithelial Cell-Matrix Interactions in the Human Intestinal Crypt. J.Signal.Transduct 2012, 248759 (2012).

22 D'Souza SE, Ginsberg MH, Plow EF. Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. Trends Biochem.Sci. 16, 246-250 (1991).

23 Bidarra SJ, Barrias CC, Fonseca KB, Barbosa MA, Soares RA, Granja PL. Injectable in situ crosslinkable RGD-modified alginate matrix for endothelial cells delivery. Biomaterials 32, 7897-7904 (2011).

24 Connelly JT, Garcia AJ, Levenston ME. Inhibition of *in vitro* chondrogenesis in RGD-modified three-dimensional alginate gels. Biomaterials 28, 1071-1083 (2007).

25 Kim SY, Oh HK, Ha JM, et al. RGD-peptide presents anti-adhesive effect, but not direct pro-apoptotic effect on endothelial progenitor cells. Arch.Biochem.Biophys. 459, 40-49 (2007).

26 Lagunas A, Comelles J, Martinez E, et al. Cell adhesion and focal contact formation on linear RGD molecular gradients: study of non-linear concentration dependence effects. Nanomedicine 8, 432-439 (2012).

27 Re'em T, Tsur-Gang O, Cohen S. The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGFbeta1-induced chondrogenesis of human mesenchymal stem cells. Biomaterials 31, 6746-6755 (2010).

28 Wang X, Yan C, Ye K, He Y, Li Z, Ding J. Effect of RGD nanospacing on differentiation of stem cells. Biomaterials 34, 2865-2874 (2013).

29 Roach P, Farrar D, Perry CC. Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. J.Am.Chem.Soc. 128, 3939-3945 (2006).

30 Pierschbacher MD, Ruoslahti E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. J.Biol.Chem. 262, 17294-17298 (1987).

31 Houseman BT, Mrksich M. The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion. Biomaterials 22, 943-955 (2001).

32 Bellis SL. Advantages of RGD peptides for directing cell association with biomaterials. Biomaterials 32, 4205-4210 (2011).

33 Santos E, Garate A, Pedraz JL, Orive G, Hernandez RM. The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells: *In vitro* and *in vivo* direct comparative study. J.Biomed.Mater.

Res.A. (2013).

34 Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. J.Biomed.Mater.Res. 60, 217-223 (2002).

35 Rowley JA, Madlambayan G, Mooney DJ. Alginate hydrogels as synthetic extracellular matrix materials. Biomaterials 20, 45-53 (1999).

36 Comisar WA, Hsiong SX, Kong HJ, Mooney DJ, Linderman JJ. Multi-scale modeling to predict ligand presentation within RGD nanopatterned hydrogels. Biomaterials 27, 2322-2329 (2006).

37 Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 210, 908-910 (1980).

38 Dai W, Saltzman WM. Fibroblast aggregation by suspension with conjugates of poly(ethylene glycol) and RGD. Biotechnol.Bioeng. 50, 349-356 (1996).

39 Jiang LY, Lv B, Luo Y. The effects of an RGD-PAMAM dendrimer conjugate in 3D spheroid culture on cell proliferation, expression and aggregation. Biomaterials 34, 2665-2673 (2013).

40 Koo LY, Irvine DJ, Mayes AM, Lauffenburger DA, Griffith LG. Co-regulation of cell adhesion by nanoscale RGD organization and mechanical stimulus. J.Cell.Sci. 115, 1423-1433 (2002).

41 Orive G, Hernandez RM, Gascon AR, Igartua M, Pedraz JL. Survival of different cell lines in alginate-agarose microcapsules. Eur.J.Pharm. Sci. 18, 23-30 (2003).

42 Sayyar B, Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Cell-matrix Interactions of Factor IX (FIX)-engineered human mesenchymal stromal cells encapsulated in RGD-alginate vs. Fibrinogen-alginate microcapsules. Artif.Cells Nanomed Biotechnol. (2013).

43 Sayyar B, Dodd M, Wen J, et al. Encapsulation of factor IX-engineered mesenchymal stem cells in fibrinogen-alginate microcapsules enhances their viability and transgene secretion. J.Tissue Eng. 3, 2041731412462018 (2012).

44 Yasuhara T, Shingo T, Muraoka K, et al. Neurorescue effects of VEGF on a rat model of Parkinson's disease. Brain Res. 1053, 10-18 (2005).

45 Neff JA, Tresco PA, Caldwell KD. Surface modification for controlled studies of cell-ligand interactions. Biomaterials 20, 2377-2393 (1999).

46 Comisar WA, Kazmers NH, Mooney DJ, Linderman JJ. Engineering RGD nanopatterned hydrogels to control preosteoblast behavior: a combined computational and experimental approach. Biomaterials 28, 4409-4417 (2007).

47 Bidarra SJ, Barrias CC, Barbosa MA, Soares R, Granja PL. Immobilization of human mesenchymal stem cells within RGD-grafted alginate microspheres and assessment of their angiogenic potential. Biomacromolecules 11, 1956-1964 (2010).

48 Evangelista MB, Hsiong SX, Fernandes R, et al. Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. Biomaterials 28, 3644-3655 (2007).

49 Li H, Frith J, Cooper-White JJ. Modulation of Stem Cell Adhesion and Morphology via Facile Control over Surface Presentation of Cell Adhesion Molecules. Biomacromolecules 15, 43-52 (2014).

Chapter 2

The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells. In vitro and in vivo direct comparative study

Journal of Biomedical Material Research Part A 102 (11), 3965-3972 (2014)

The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells. *In vitro* and *in vivo* direct comparative study

Edorta Santos[1,2,a], Ane Garate[1,2,a], José Luis Pedraz[1,2], Gorka Orive[1,2], Rosa María Hernández[1,2]

1NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Vitoria, Spain.
2Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria, Spain.
aThese two authors contributed equally to this work.

ABSTRACT

The inclusion of the tripeptide RGD (Arg-Gly-Asp) in otherwise inert biomaterials employed for cell encapsulation has been observed to be an effective strategy to provide the immobilized cells with a more suitable microenvironment. However, some controversial results collected during the last years, especially *in vivo*, have questioned its effectiveness. Here, we have studied the behavior of C_2C_{12} myoblasts immobilized in alginate-poly-L-lysine-alginate (APA) microcapsules with different densities of RGD. The use of these microcapsules offer the advantage of avoiding native proteins influence permitting to establish direct comparisons between *in vitro* and *in vivo* assays. Results suggest that RGD-modified matrices provide higher dynamism, achieving therapeutically more active biosystems not only *in vitro*, but also *in vivo*. The highest functionality of the immobilized cells *in vitro* was obtained with the lowest RGD density. However, higher RGD densities were required *in vivo* to obtain the same effects observed *in vitro*. Altogether, these results suggest the lack of *in vitro-in vivo* correlation when cell behavior is evaluated within different RGD-tailored cell-loaded scaffolds.

Keywords
Microcapsule, RGD, scaffold, ligand density, microenvironment

1. Introduction

During the last decade, the behavior of entrapped cells and the functionality of 3D biosystems have become a major focus of interest in the field of therapeutic cell encapsulation, leading to increasingly sophisticated scaffold designs that provide the encapsulated cells with a more suitable and natural microenvironment [1-3]. One of the most employed molecules for such aim is the tripeptide arginine-glicine-aspartate (RGD), the principal integrin-binding domain present in natural adhesion proteins of the extracellular matrix (ECM) such as fibronectin, vibronectin or fibrinogen. The inclusion of RGD in otherwise inert biomaterials promotes the adhesion and survival of encapsulated cells, leading to mechanically optimized cell-based scaffolds, which enhance the long-term functionality of the cell-based biosystems [4-7]. Furthermore, the use of this short amino acid sequence offers several advantages over the previous mentioned native ECM molecules including the low risk of immune reactivity, the tight control over ligand presentation or the straightforward synthesis [8-10].

However, despite the demonstrated potency of this peptide sequence as bioactive molecule, recent investigations have shed controversial results concerning the effect of this adhesion moiety, opening an extended debate about its use [11-14]. While *in vitro* studies have confirmed the effectiveness of RGD peptides in enhancing cell function through the regulation of integrin-mediated signaling pathway, *in vivo* studies have been shown to be more variable [15-17]. This fact makes the so far used *in vitro* methods unreliable reporters of *in vivo* activity and, thereby, highlights the need for more *in vivo* studies in order to bring this therapy towards clinical reality. In this sense, researchers in the field are currently discussing the diverse factors that may influence in this lack of consistency between *in vitro* and *in vivo* results, including the background produced by the serum proteins adsorbed in the matrix [18,19] or the synergistic effect mediated by the different physicochemical cues coming from the surrounded microenvironment [20,21]. Besides, the use of different RGD ligand types, densities or presentation patterns, may be additional parameters that introduce variability and confound the interpretation of the obtained results [22-24]. In addition, the effect of all these

variables is much probably cell type dependent [25,26]. In fact, although numerous studies have been carried out to gain insight into the repercussion of the tripeptide RGD, the drawn conclusions are diverse and unalike [27,28]. The discrepancies regarding the therapeutic benefits of RGD as optimal strategy to modify biomaterials still continue, increasing the need for collecting these parameters in a unique comparative study.

The inability of alginate to support cell interaction and attachment of mammalian cells, together with the low protein adsorptive capacity of its hydrogels, makes this polymer an ideal platform for this type of study [29,30]. Moreover, one of the most studied 3D alginate scaffolds, namely alginate-poly-l-lysine-alginate (APA) microcapsules, represents an especially attractive model, as the semipermeable PLL membrane avoids/prevents the possible diffusion of serum proteins from the surrounding microenvironment [31]. Thus, it is possible to remove the "background noise" and isolate the variables under study. In addition, this biosystem, due to its biocompatibility and biosafety, offers reliable translation from *in vitro* to *in vivo* studies, allowing facile and direct comparison between both [32-34].

In the present work, C_2C_{12} myoblasts genetically engineered to secrete EPO were encapsulated in APA microcapsules with different RGD densities to further analyze the number of viable cells per capsule, the proliferation and the secretion of therapeutic factor either *in vitro* or *in vivo*. To the best of our knowledge, this is the first report involving in a unique and comprehensive study some of the prime factors that may have influence in the effect of RGD on the encapsulated cells, providing comparative data between results obtained *in vitro* and *in vivo*. This is intended to shed some light on the existing debate about this issue in the field.

2. Materials and methods

2.1. Cell culture

C3H-mouse C_2C_{12} myoblasts, genetically modified to deliver EPO, were kindly provided by the Institute des Neurosciences (Ecole Polytechnique Federale of Lausanne, Switzerland). The cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% foetal bovine serum (FBS),

L-glutamine to a final concentration of 2 mM, 4.5 g/L glucose and 1% antibiotic/antimycotic solution. Cells were plated in T-flasks, maintained at 37 ºC in a 5% CO_2/95% air atmosphere and passaged every 2-3 days. All reagents were purchased from Gibco BRL (Life technologies, Spain).

2.2. Incorporation of adhesion molecules into alginate

Alginate was chemically modified by the aqueous carbodiimide chemistry. A water soluble carbodiimide, (1-ethyl-dimethylaminopropyl) carbodiimide (EDC), was used to form amide linkages between amine containing molecules and the carboxylate moieties on the alginate polymer backbone, with a reaction efficiency of approximately 80% [8]. The total number of RGD peptides per alginate chain, defined as the degree of substitution (DS) [10], was altered by varying the concentration of RGD peptides in the coupling reaction, obtaining four different types of alginate: DS 0 (No modified alginate), DS 1 (0.112 mM), DS 5 (0.5 mM) and DS 10 (1.12 mM).

2.3. Cell microencapsulation

C_2C_{12} myoblasts genetically modified to release EPO were incorporated into 3D alginate-poly-l-lysine-alginate (APA) microcapsules using an electrostatic droplet generator with brief modifications of the procedure designed by Lim and Sun [35]. Briefly, cells were harvested from monolayer cultures using trypsin- EDTA (Life technologies), filtered through a 40 μm pore mesh and suspended in four different solutions of 1.5% (w/v) sodium alginate (DS 0, DS 1, DS 5, DS 10) at $5x10^6$ cells/ml density. The resulted suspensions were extruded in a sterile syringe through a 0.35 mm needle at a 5.9 mL/h flow rate using a peristaltic pump. The resulting alginate particles were collected in a 55mM $CaCl_2$ solution and maintained under agitation for 15 min after the end of the process to ensure complete gelation of all the beads. Then, the obtained particles were suspended in 0.05% PLL solution for 5 min, washed twice with 10 mL of manitol 1% and coated again with another layer of 0.1% alginate for 5 min. All the process was carried out under aseptic conditions at room temperature, and resulting microcapsules were cultured in complete medium at 37 ºC in a 5% CO_2/95% air atmosphere standard incubator. Ultra pure low-viscosity high guluronic acid alginate (UPLVG) was purchased from FMC Biopolymer, Norway, and poly-l-lysine

(PLL hidrobromide Mw 15 000–30 000 Da) was obtained from Sigma Aldrich (St. Louis,MO, USA).

2.4. Cell Viability

Cells entrapped into APA microcpasules were dyed with the LIVE/DEAD kit (Life technologies) following manufacturer's indications. After 30 min, fluorescence micrographs were taken using an epi-fluorescence microscope (Nikon TSM).

2.5. Quantification of the total number of living cells per capsule

In order to determine the exact number of living cells quantitatively, enclosed cells were firstly de-encapsulated with 500 µg/mL of alginate lyase (Sigma-Aldrich). LIVE/DEAD kit (Life technologies) was used to differentiate living and dead cells. After incubation of samples for 20 min at room temperature and protected from light, cells were counted by means of flow cytometry (BD FACSCalibur) using Trucount Tubes (BD). All samples were assayed in triplicate for all groups, and obtained values are shown as mean of 3 independent samples ± S.D per study group.

2.6. Measurement of EPO secretion

Encapsulated C_2C_{12} myoblasts supernatants were assayed for EPO secretion using the Quantikine IVD Human Erythropoietin ELISA Kit purchased from R&D Systems (Minneapolis, MN). Standards and samples were run in duplicate according to the procedure specified in the kit. The EPO secretion of the equivalent of 1.5×10^4 cells/mL was measured for a 24 h release period in triplicate per study group, and results are expressed as mean ± S.D.

2.7. Cell proliferation assay

The equivalent of 2×10^4 cells/100 µL (≈100 microcapsule/ well) was placed into each well of 96-well plate. All groups were incubated with complete medium supplemented with 10% FBS except the negative control group, which was incubated with starving medium supplemented with 0.1% FBS. After 24 h, the encapsulated cells were incubated in the presence of 10 µM BrdU for an additional day, except non-specific binding control group. The third day cells were de-encapsulated using 500 µg/mL of alginate lyase (Sigma-Aldrich) and assayed for BrdU uptake using Cell Proliferation Biotrak ELISA System (Amersham, NJ, USA) following manufacturer's indications.

Absorbance measurements of the non-specific binding control group (without BrdU) were subtracted from the rest of the groups, and results were normalized with the corresponding negative control (microcapsules incubated with 0.1 % FBS) for each experiment. Data are shown as mean of 5 independent samples ± S.D per study group.

2.8. Cell morphology. Determination of F actin

Microcapsules (100 µl of capsules) were fixed in 4 % paraformaldehyde, washed in pre-warmed DPBS and permeabilized by 0.1 % Triton X-100 for 10 minutes. The cytoskeleton of encapsulated cells was stained with Alexa Fluor 488 phalloidin, a volume of 15 µL methanolic stock solution in 200 µl DPBS, for 30 min in the dark (Life technologies) containing 1 % bovine serum albumin to reduce nonspecific background. The nucleus of the cells were dyed with Hoechst (1 µg/ml) and the samples were analyzed by inverted confocal microscopy (Olympus Fluoview 500 Confocal Microscopy).

2.9. Microcapsule implantation and retrieval to evaluate explanted microencapsulated cells

Animal studies were carried out according to the ethical guidelines established by our Institutions, under an approved animal protocol (241/2012). Adult female Balb/c mice (n=6 per group) were anesthetized by isoflurane inhalation, and implanted subcutaneously with a total volume of 300 µL of cell-loaded microcapsules ($5x10^6$ cells/mL) suspended in PBS using a 20-gauge catheter (Nipro; Nissho Corp, Belgium). Animals were housed in specific pathogen free facility under controlled temperature and humidity with a standardized 12 h light/dark cycle and had access to food and water ad libitum. At day 15 and 30 after implantation, 3 animals from each group were sacrificed and capsules were explanted. Briefly, a mix of collagenase H (2mg/ml) (Roche Diagnostics, Germany) and hyaluronidase (1mg/ml) (Sigma, St. Louis, USA) was prepared using DMEM. This enzyme solution was filtered-sterilized prior to use. Using 50 mL tubes, 5-6 mL of disgregation solution was added to around 3-4 mL of a microcapsule aggregate. Once tubes were carefully sealed, they were incubated in a shaker bath at 37 ºC at 100 rpm for 4 h. Once the surrounding tissue was disaggregated, the solution in the tubes was filtered using 40

µm pore size filters to recover tissue-free capsules.

2.10. Statistical analysis

Data are presented as mean ± S.D. One-way ANOVA and post-hoc test were used in multiple comparisons. The Bonferroni or Tamhane post-hoc test was applied according to the result of the Levene test of homogeneity of variances. In the case of non-normally distributed data, Mann-Whitney non-parametric analysis was used. All statistical computations were performed using SPSS 20 (SPSS, Inc., Chicago, IL).

3. Results

3.1. Cell viability in vitro

In order to carry out a thorough characterization of encapsulated myoblasts *in vitro*, we first evaluated the number of living cells/cap by using flow cytometry. In this viability assay, slight intergroup differences were observed with a low statistical significance after 30 days of encapsulation without a clear trend during all the study (Fig. 1A). Moreover, the cells entrapped in all type of microcapsules showed a lower viability on the last day than by day 15. Fluorescence

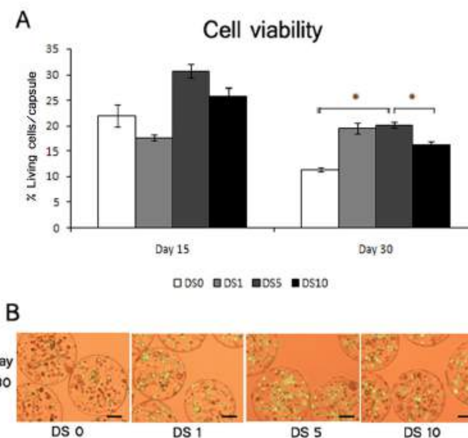


Figure 1. (A) *In vitro* percentage of living cells after 15 and 30 days of encapsulation. The number of living cells obtained for the day 0 was considered as 100% in each microcapsules group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). (B) *In vitro* Fluorescence micrographs taken by day 30. Scale bars = 100 µm.

micrographs, taken in parallel to flow cytometry assays, provided further evidence on our observations showing a similar green fluorescence in all type of elaborated microcapsules (Fig. 1B).

3.2. EPO secretion and cell proliferation in vitro

EPO secretion and BrdU uptake were analyzed in order to evaluate the functionality, proliferative capacity and

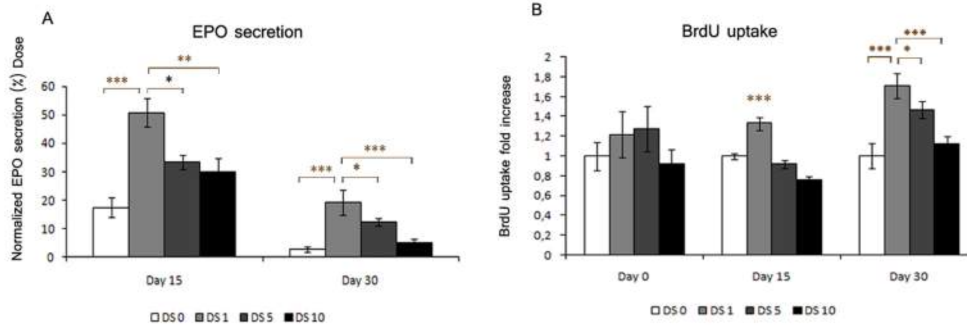


Figure 2. (A) *In vitro* EPO secretion after 15 and 30 days of encapsulation. Therapeutic factor secretion levels obtained for the day 0 were considered as 100% in each microcapsule group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). (B) *In vitro* BrdU uptake after 0, 15 and 30 days of encapsulation. The results were normalized with those obtained with DS 0 group each day. Bar graphs symbolize the mean ± S.D (n=5). Statistical significance *p < 0.05, **p < 0.01 and ***p < 0.001.

behavior of immobilized cells. Overall, the RGD-coupled alginate microcapsules maintained higher values of therapeutic factor secretion than microcapsules without RGD, independently of their substitution degree. However, DS 1 was the group with the highest level of therapeutic factor secretion after 15 and 30 days of encapsulation, showing normalized EPO secretion values of 50.9 % ± 4.9 and 19.2 % ± 4.2 respectively. The differences were more evident when this group was compared to DS 0 and DS 10 groups (p<0.001) than to DS 5 group (p<0.05) (Fig. 2A). As expected, the results of proliferation activity indicated the highest DNA synthesis for the DS 1 group at day 30, as revealed by the 1.7 ± 0.1-fold higher BrdU uptake. The differences were even more evident when this group was compared to DS 0 and DS 10 groups (p<0.001) than to DS 5 group (p<0.05) (Fig. 2B). These results come along with those obtained in the therapeutic factor secretion assay.

3.3. Cell morphology. Determination of F-actin

In order to obtain more detailed information regarding cell-ECM interaction, we next assessed the morphology of immobilized cells by confocal microscopy after staining the

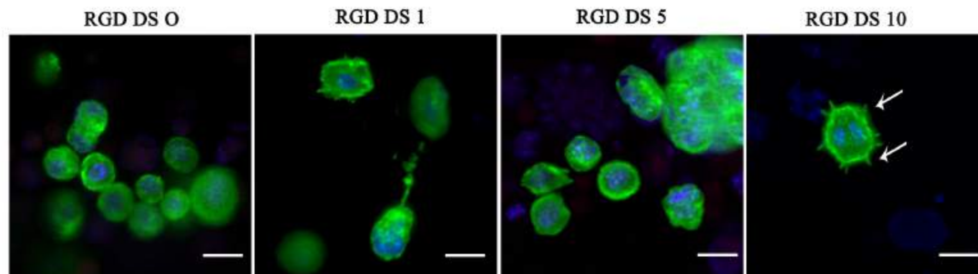


Figure 3. Cytoskeleton organization of myoblasts encapsulated in microcapsules elaborated with four different types of alginate *in vitro*. The cells inside APA microcapsules were stained with phalloidin Alexa Fluor 488 for F-actin (green) and Hoechst (blue) for nucleus. Scale bars = 20 µm.

F-actin filaments with phalloidin Alexa Fluor 488. Photographs shown in Figure 3 demonstrate the presence of filopodia-like membrane extensions in the case of cells immobilized within RGD-coupled alginate matrices, being more prominent as RGD density increased. Contrariwise, microcapsules without RGD retained the typical round shape in enclosed cells with no detectable cytoplasm extensions.

3.4. Cell viability in vivo

In a second set of experiments, the effectiveness of RGD was assessed *in vivo* in order to study the influence of the physiological environment on the immobilized cells. Here, no statistical differences among the groups were obtained after 30 days of the study (Fig. 4A). Unlike *in vitro* study, the viability of immobilized cells *in vivo* increased

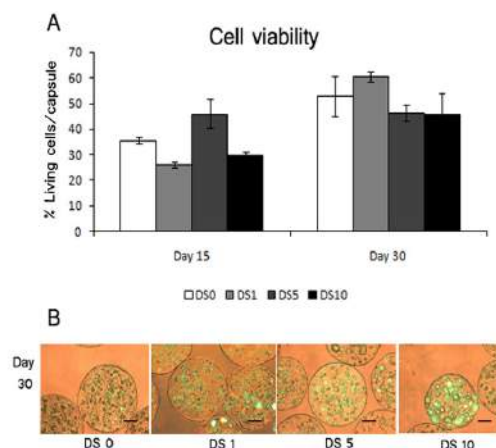


Figure 4. (A) *In vivo* percentage of living cells after 15 and 30 days of encapsulation. The number of living cells obtained for the day 0 was considered as 100% in each microcapsules group, and all values were expressed in function of this percentage. (B) *In vivo* Fluorescence micrographs taken by day 30. Bar graphs symbolize the mean ± S.D (n=3). Scale bars = 100 µm.

over the course of 30 days. Fluorescence micrographs, taken in parallel to flow cytometry showed a similar green fluorescence in all type of microcapsules reflecting the quantitative data obtained in the previous assay (Fig. 4B).

3.5. EPO secretion and cell proliferation in vivo

In contrast to *in vitro* results, where the DS 1 group showed the highest secretion of the therapeutic factor, DS 5 group showed the highest EPO secretion *in vivo* by day 30, showing a normalized value of 35.9 % ± 5.8. This value resulted statistically significant when compared to DS 0 and DS 1 groups (p<0.001) (Fig. 5A). In accordance with the previous assay, the DS 5 group maintained the highest DNA synthesis, as revealed by 4.2 ± 0.5-fold higher BrdU uptake with respect to DS 0 group (p<0.001) (Fig. 5B).

3.6. Differences between in vitro and in vivo studies by day 30

For a better understanding/ comprehension of the data obtained in this study, we elaborated a representative graphical analysis highlighting the differences between *in vitro* and *in vivo* assays (Fig. 6). For this purpose, the data obtained in each study with the DS 0 group was compared with the groups which contained different substitution

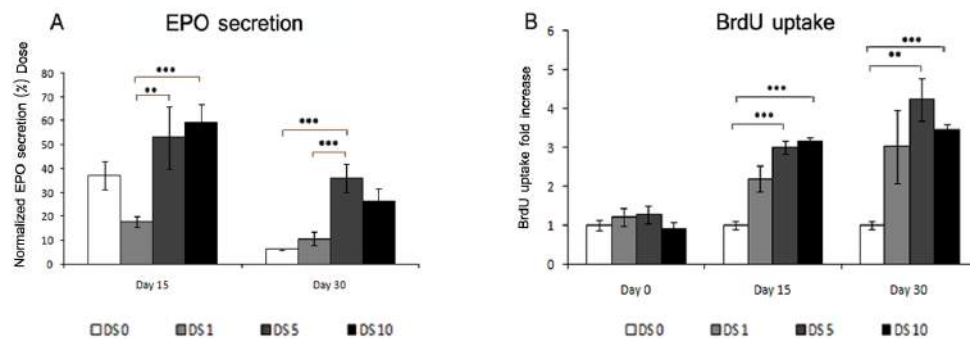


Figure 5. (A) *In vivo* EPO secretion after 15 and 30 days of encapsulation. Therapeutic factor secretion levels obtained for the day 0 were considered as 100% in each microcapsule group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). (B) BrdU uptake of myoblasts after 0, 15 and 30 days of encapsulation. The results were normalized with those obtained with DS 0 group each day. Bar graphs symbolize the mean ± S.D (n=5). Statistical significance **p < 0.01 and ***p < 0.001.

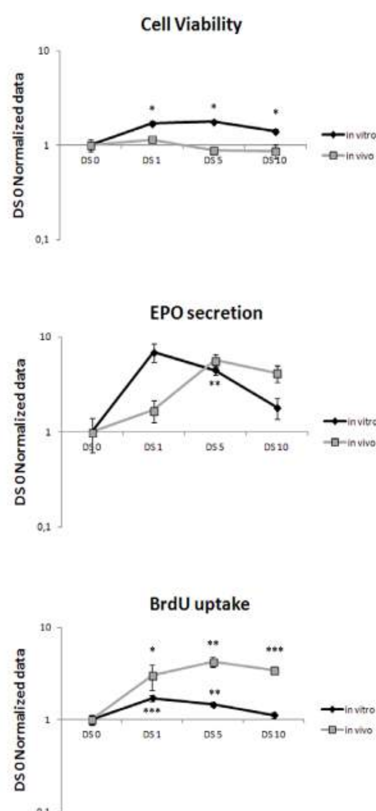


Figure 6. Direct comparison between *in vitro* and *in vivo* studies showing the synergistic effect of the RGD density and the microenvironment on cell viability, EPO secretion and BrdU uptake. The data obtained in either *in vitro* or *in vivo* studies were normalized against their respective DS 0 control group in order to compare the behavior of encapsulated cells in these two microenvironments in function of RGD density (DS 1, DS 5 and DS 10). Bar graphs symbolize the mean ± S.D (Standard deviation is within the size of the symbols in the graph). Statistical significance *p < 0.05, **p < 0.01 and ***p < 0.001; DS 0 vs other groups (DS1, DS5 and DS 10).

degrees of RGD in order to observe the effect of different ligand densities on the behavior of entrapped cells compared to non-modified alginate. Although the viability assays pointed out no statistical significant influence of RGD matrices on the number of living cells/cap either *in vitro* or *in vivo*, EPO secretion and proliferation profiles clearly showed that *in vitro*, DS 1 was the group which demonstrated the most prominent effect on encapsulated cell. *In vivo*, this effect resulted more noticeable in the DS 5 group.

4. Discussion

In recent years increasingly sophisticated and tailored 3D bioscaffolds are being designed to compensate at least in part for the missing natural microenvironment of encapsulated cells. The RGD sequence, being the minimal adhesion ligand domain present in some ECM proteins, is one of the most commonly used molecule in this field due to its proved positive biological impact on the behavior of immobilized cells. However, some controversial results collected during the last years, especially *in vivo*, have questioned its effectiveness, opening an extended debate about its use. To address this, we immobilized

erythropoietin (EPO)-releasing C_2C_{12} myoblasts within APA microcapsules in order to study the effect of different RGD densities both *in vitro* and *in vivo*, and with the aim of shedding some light on this topic of discussion.

As described in the literature, the adsorption of serum proteins – including integrin-binding native proteins such as fibronectin or vibronectin – into the biomaterials, may produce undesirable effects that lead to an increasing variability between *in vitro* and *in vivo* studies [36-39]. Importantly, the APA microcapsules employed in this study offer the advantage of avoiding such native proteins influence. This is given due to two main reasons: the low capacity of alginate gels to adsorb proteins; and the physical barrier provided by the semipermeable membrane of the microcapsules, which prevents the inward diffusion of serum proteins with molecular weights above the cut-off usually established in 70 KDa [29,31,40]. Thus, the employment of APA microcapsules in the present study permited the observation of RGD effects in an isolated way, removing the background of native proteins that may mask, at least in part, the effectiveness of RGD to induce different cell responses.

It is well known that life and death decisions at the cellular level are profoundly influenced by the proteins of ECM [41,42]. Indeed, extensive studies have proved the capability of RGD moiety to promote vital cellular functions such as adhesion, migration, survival, proliferation, differentiation, morphogenesis and gene expression by means of integrin-mediated signaling pathways [43-45].

In this work, the *in vitro* assays showed that although the number of living cells/cap did not change too much with the inclusion of different RGD densities in alginate matrices (Fig. 1), the cells enclosed within RGD-coupled alginates had more capacity to proliferate and secrete therapeutic factors (Fig. 2A-2B). In the current study, unlike many other works in the field, the viability was not evaluated by methods based on metabolic activity, achieving more accurate data. Thus, we also had the opportunity to know better the exact number of living cells per capsule and to show the necessity to improve the obtained worrysome results in the future. Moreover, although the viability was similar in both days (Fig. 1) the EPO secretion by day 30 was too much lower (Fig. 2A). This could be explained by the fact that although in the cytometry

assay the cells might dye with green fluorescence as a living cell, its metabolic activity could be reduced. Anyway, the obtained results lead to the hypothesis that the living myoblasts entrapped in the presence of RGD were more active than those enclosed within non-modified alginate scaffolds which gives rise to more dynamic and functional biosystems. Such dynamism would cause higher rates of cell proliferation and cell death, resulting in a continuous replacement and renewal of the cell content. This phenomenon would have a notorious impact in therapeutic cell encapsulation, as newly formed "fresh" cells would contribute to the increase of therapeutic activity (in terms of either duration or quality), while preventing the biosystem from aging.

Although further studies are required to analyze the biology and mechanism of the cell-matrix interactions, the analyses of actin filaments *in vitro* suggested that immobilized cells were able to establish interaction sites with alginate in microcapsules containing RGD, whereas cells enclosed within alginate matrices without RGD remained round (Fig. 3). Even if these filopodia-like extensions, indicators of cell spreading, were more prominent as RGD density increased, the highest proliferation and EPO secretion were obtained with the lowest RGD density (DS 1 microcapsules). Our findings come along with other previously reported results in the literature which revealed that intermediate levels of the triamino sequence are optimal to obtain the maximum proliferation rate of myoblasts *in vitro* [46]. In fact, as observed in other studies, while an optimal cell spreading was obtained with high densities of RGD, the maximum proliferation required lower adhesion ligand presentation [47]. This phenomenon was explained by assuming that the strong adhesions resulting from many bound receptors may impede cell division, producing an inhibitory effect when the employed densities of RGD are too high [48]. However, this theory is still no clear, and as mentioned previously, the RGD moiety may promote other vital cellular functions such as differentiation of enclosed cells hindering the proliferation of the cells.

In the current study, a total of 30 days of follow-up were required to achieve notorious differences between microcapsules elaborated with different densities of RGD. This may explain some of the discrepancies described in previous studies in which the time intervals

evaluated were lower [25,26]. Indeed, depending on the specific application or study, the multiple effects of RGD may be expressed at different times, according also to the scaffold model and the cell type used. The results obtained in the present work are specific to C_2C_{12} myoblasts, one of the most studied cell line in the field. It is known that the RGD density of the matrices and the microenvironment may affect in a different way depending on the cell type [26]. Thus, future efforts should be focused on finding the optimal density of RGD for each cell type.

We next moved on to *in vivo* assays in order to test the influence of a physiological microenvironment on cells enclosed within RGD-enriched matrices. When the microcapsules were retrieved from the animals, there was no evidence of inflammation process neither differences on the volume or adherence in all types of microcapsules elaborated with different RGD densities (data not shown). With the aim of isolating this variable, we repeated the same experimental procedure carried out *in vitro*, and the differences between both types of studies by day 30 were collected in the Figure 6. Higher RGD densities seem to be required *in vivo* to obtain the same effects observed *in vitro*. Graph curves for all assayed parameters revealed clearly that enclosed cells reflected almost the same behavior shown *in vitro* but displaced to higher densities of RGD. This also includes the inhibitory effect produced at the highest RGD density (DS 10). In the particular case of proliferation, it must be taken into account that the inclusion of RGD led to a higher proliferation activity in all types of microcapsules *in vivo*, compared to the lower values obtained *in vitro*. These differences may be attributed to the complexity provided by the *in vivo* body fluids to the microenvironment where microcapsules reside, which may also influence the final outcome of RGD on encapsulated cells. In fact, it is well known that several growth factors and hormones may alter the integrin expression of cells, and that their receptors cooperate with integrins in the regulation of adhesion-mediated signaling networks [20,21,49]. Therefore, special attention must be paid to the synergistic effect of the molecules coming from surrounding microenvironment, as this latter may vary according to the implantation site.

Some important parameters for the design of biomimetic biomaterials such as optimal RGD density and the influence

of the surrounded microenvironment are presented in this study. Although further investigation are needed to define the molecular and cellular basis of these observations, these types of screening studies provide meaningful information in order to explore the complexity entailed by cell-ECM interaction. Likewise, future studies should be focused on studying the efficacy of RGD taking into account other parameters such as cell type or implantation site in the animal.

5. Conclusions

This work adds further information to the existing debate about the therapeutic benefits resulting from the use of RGD. APA microcapsule design demonstrated to be a suitable model for the study of cell-RGD interaction due to its ability to exclude the influence derived from the adsorption of serum proteins. This also permitted to establish direct comparisons between *in vitro* and *in vivo* assays. RGD-modified matrices showed a higher dynamism to promote the renewal and replacement of the cell content and thereby achieve therapeutically more active biosystems. Finally, the present study showed clear differences between *in vitro* and *in vivo* assays, emphasizing the importance of the synergistic effect caused by the surrounding microenvironment and the difficulty to extrapolate *in vitro* results to *in vivo* reality.

Acknowledgements

Authors would like to thank Ricardo Andrade for technical help with confocal microscopy. The authors also gratefully acknowledge the support to research cell microencapsulation from the “Ministerio de Ciencia e Innovación”, University of the Basque Country UPV/EHU (UFI11/32) and FEDER funds (SAF2008-03157). A. Garate thanks the “Gobierno Vasco (Departamento de Educación, Universidades e Investigación)” for the Ph.D fellowship.

References

1 Sayyar B, Dodd M, Wen J, et al. Encapsulation of factor IX-engineered mesenchymal stem cells in fibrinogen-alginate microcapsules enhances their viability and transgene secretion. J.Tissue Eng. 3, 2041731412462018 (2012).

2 Karoubi G, Ormiston ML, Stewart DJ, Courtman DW. Single-cell hydrogel encapsulation for enhanced survival of human marrow stromal cells. Biomaterials 30, 5445-5455 (2009).

3 Kong HJ, Smith MK, Mooney DJ. Designing alginate hydrogels to maintain viability of immobilized cells. Biomaterials 24, 4023-4029 (2003).

4 Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 24, 4385-4415 (2003).

5 Hsiong SX, Huebsch N, Fischbach C, Kong HJ, Mooney DJ. Integrin-adhesion ligand bond formation of preosteoblasts and stem cells in three-dimensional RGD presenting matrices. Biomacromolecules 9, 1843-1851 (2008).

6 Orive G, De Castro M, Kong HJ, et al. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. J.Control.Release 135, 203-210 (2009).

7 Salinas CN, Anseth KS. The influence of the RGD peptide motif and its contextual presentation in PEG gels on human mesenchymal stem cell viability. J.Tissue Eng. Regen.Med. 2, 296-304 (2008).

8 Rowley JA, Madlambayan G, Mooney DJ. Alginate hydrogels as synthetic extracellular matrix materials. Biomaterials 20, 45-53 (1999).

9 Santos E, Hernandez RM, Pedraz JL, Orive G. Novel advances in the design of three-dimensional bio-scaffolds to control cell fate: translation from 2D to 3D. Trends Biotechnol. 30, 331-341 (2012).

10 Comisar WA, Hsiong SX, Kong HJ, Mooney DJ, Linderman JJ. Multi-scale modeling to predict ligand presentation within RGD nanopatterned hydrogels. Biomaterials 27, 2322-2329 (2006).

11 Bellis SL. Advantages of RGD peptides for directing cell association with biomaterials. Biomaterials 32, 4205-4210 (2011).

12 Barker TH. The role of ECM proteins and protein fragments in guiding cell behavior in regenerative medicine. Biomaterials 32, 4211-4214 (2011).

13 Collier JH, Segura T. Evolving the use of peptides as components of biomaterials. Biomaterials 32, 4198-4204 (2011).

14 Williams DF. The role of short synthetic adhesion peptides in regenerative medicine; the debate. Biomaterials 32, 4195-4197 (2011).

15 Ferris DM, Moodie GD, Dimond PM, Gioranni CW, Ehrlich MG, Valentini RF. RGD-coated titanium implants stimulate increased bone formation *in vivo*. Biomaterials 20, 2323-2331 (1999).

16 Eid K, Chen E, Griffith L, Glowacki J. Effect of RGD coating on osteocompatibility of PLGA-polymer disks in a rat tibial wound. J.Biomed. Mater.Res. 57, 224-231 (2001).

17 Alsberg E, Anderson KW, Albeiruti A, Rowley JA, Mooney DJ. Engineering growing tissues. Proc.Natl.Acad.Sci.U.S.A. 99, 12025-12030 (2002).

18 Allen LT, Tosetto M, Miller IS, et al. Surface-induced changes in protein adsorption and implications for cellular phenotypic responses to surface interaction. Biomaterials 27, 3096-3108 (2006).

19 Roach P, Farrar D, Perry CC. Surface

tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. J.Am.Chem.Soc. 128, 3939-3945 (2006).

20 Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. J.Biol.Chem. 264, 380-388 (1989).

21 Sinha RK, Tuan RS. Regulation of human osteoblast integrin expression by orthopedic implant materials. Bone 18, 451-457 (1996).

22 Pierschbacher MD, Ruoslahti E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. J.Biol.Chem. 262, 17294-17298 (1987).

23 Shin H, Jo S, Mikos AG. Biomimetic materials for tissue engineering. Biomaterials 24, 4353-4364 (2003).

24 Lagunas A, Comelles J, Martinez E, et al. Cell adhesion and focal contact formation on linear RGD molecular gradients: study of non-linear concentration dependence effects. Nanomedicine 8, 432-439 (2012).

25 Connelly JT, Garcia AJ, Levenston ME. Inhibition of *in vitro* chondrogenesis in RGD-modified three-dimensional alginate gels. Biomaterials 28, 1071-1083 (2007).

26 Re'em T, Tsur-Gang O, Cohen S. The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGFbeta1-induced chondrogenesis of human mesenchymal stem cells. Biomaterials 31, 6746-6755 (2010).

27 Bidarra SJ, Barrias CC, Fonseca KB, Barbosa MA, Soares RA, Granja PL. Injectable in situ crosslinkable RGD-modified alginate matrix for endothelial cells delivery. Biomaterials 32, 7897-7904 (2011).

28 Kim SY, Oh HK, Ha JM, et al. RGD-peptide presents anti-adhesive effect, but not direct pro-apoptotic effect on endothelial progenitor cells. Arch.Biochem.Biophys. 459, 40-49 (2007).

29 Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog.Polym.Sci. 37, 106-126 (2012).

30 Machida-Sano I, Ogawa S, Ueda H, Kimura Y, Satoh N, Namiki H. Effects of composition of iron-cross-linked alginate hydrogels for cultivation of human dermal fibroblasts. Int.J.Biomater. 2012, 820513 (2012).

31 Leung A, Nielsen L, Trau M, Timmins N. Tissue transplantation by stealth-coherent alginate microcapsules for immunoisolation. Biochem Eng J 48, 337-47 (2010).

32 Orive G, Tam SK, Pedraz JL, Halle JP. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. Biomaterials 27, 3691-3700 (2006).

33 Pawar SN, Edgar KJ. Alginate derivatization: A review of chemistry, properties and applications. Biomaterials 33, 3279-3305 (2012).

34 Ponce S, Orive G, Hernandez R, et al. Chemistry and the biological response against immunoisolating alginate-polycation capsules of different composition. Biomaterials 27, 4831-4839 (2006).

35 Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 210, 908-910 (1980).

36 Aota S, Nagai T, Yamada KM. Characterization of regions of fibronectin besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using site-directed mutagenesis. J.Biol.Chem. 266, 15938-15943 (1991).

37 Woods A, Couchman JR, Johansson S, Hook M. Adhesion and cytoskeletal organisation of fibroblasts in response to fibronectin fragments. EMBO J. 5, 665-670 (1986).

38 Sawyer AA, Hennessy KM, Bellis SL. Regulation of mesenchymal stem cell attachment and spreading on hydroxyapatite by RGD peptides and adsorbed serum proteins. Biomaterials 26, 1467-1475 (2005).

39 Sawyer AA, Hennessy KM, Bellis SL. The effect of adsorbed serum proteins, RGD and proteoglycan-binding peptides on the adhesion of mesenchymal stem cells to hydroxyapatite. Biomaterials 28, 383-392 (2007).

40 Houseman BT, Mrksich M. The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion. Biomaterials 22, 943-955 (2001).

41 Stupack DG, Cheresh DA. Get a ligand, get a life: integrins, signaling and cell survival. J.Cell.Sci. 115, 3729-3738 (2002).

42 Stupack DG, Cheresh DA. Apoptotic cues from the extracellular matrix: regulators of angiogenesis. Oncogene 22, 9022-9029 (2003).

43 Arnaout MA, Mahalingam B, Xiong JP. Integrin structure, allostery, and bidirectional signaling. Annu.Rev.Cell Dev.Biol. 21, 381-410 (2005).

44 Barczyk M, Carracedo S, Gullberg D. Integrins. Cell Tissue Res. 339, 269-280 (2010).

45 Evangelista MB, Hsiong SX, Fernandes R, et al. Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. Biomaterials 28, 3644-3655 (2007).

46 Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. J.Biomed.Mater.Res. 60, 217-223 (2002).

47 Neff JA, Tresco PA, Caldwell KD. Surface modification for controlled studies of cell-ligand interactions. Biomaterials 20, 2377-2393 (1999).

48 Comisar WA, Kazmers NH, Mooney DJ, Linderman JJ. Engineering RGD nanopatterned hydrogels to control preosteoblast behavior: a combined computational and experimental approach. Biomaterials 28, 4409-4417 (2007).

49 Plopper GE, McNamee HP, Dike LE, Bojanowski K, Ingber DE. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol.Biol.Cell 6, 1349-1365 (1995).

Chapter 3

Assessment of the behavior of MSCs immobilized in biomimetic alginate microcapsules

Assessment of the behavior of MSCs immobilized in biomimetic alginate microcapsules

Ane Garate[1,2], Jesús Ciriza[1,2], Javier G Casado[3], Rebeca Blázquez[3] , José Luis Pedraz[1,2] , Gorka Orive[1,2], Rosa María Hernández[1,2]

1NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Vitoria, Spain.
2Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria, Spain.
3Stem Cell Therapy Unit "Jesús Usón", Minimally Invasive Surgery Centre, Cáceres, Spain.

ABSTRACT

The combination of mesenchymal stem cells (MSCs) and biomimetic matrices for cell-based therapies has lead to enormous advances, including the field of cell microencapsulation technology. In the present work, we have evaluated the potential of genetically modified MSCs as a vehicle of drug delivery systems immobilized in alginate microcapsules with different RGD densities. Results demonstrated that the microcapsules represent a suitable platform for D1-MSC encapsulation since cell immobilization into alginate matrices does not affect their main characteristics. The *in vitro* study showed a higher activity of D1-MSCs when they are immobilized in RGD-modified alginate microcapsules, obtaining the highest therapeutic factor secretion with low and intermediate densities of the bioactive molecule. In addition, the inclusion of RGD increased the differentiation potential of immobilized cells upon specific induction. However, subcutaneous implantation did not induce differentiation of D1-MSCs towards any lineage remaining at an undifferentiated state *in vivo*.

Keywords

Biomimetic, microcapsule, D1 Mesenchymal stem cells (D1-MSCs), cell behavior, matrices

1. Introduction

Cell microencapsulation technology has been employed as an appropriate system for sustained delivery of diverse therapeutic factors in recent years [1-3]. This cell-based therapy consists in the immobilization of genetically modified cells into a polymeric matrix usually surrounded by a semipermeable membrane. The design allows the diffusion of nutrients and oxygen, both essential for the survival of encapsulated cells, while at the same time protects the implanted cells from the host immune response, avoiding the entrance of high molecular weight immune system components such as immunoglobulins and immune cells [4,5]. Since the first approach carried out more than 30 years ago with the encapsulation of pancreatic islets in alginate microcapsules [6], several strategies have been developed giving rise to enormous advances in the field [7-9].

The biomimetization of polymers in which cells are immobilized is an essential goal on the progress in cell-based systems [10,11]. Despite the extensive use of alginate in cell microencapsulation technology, this inert polymer is unable to provide cell-matrix interactions and inherently lacks mammalian cell-adhesivity [12,13]. The inclusion of extracellular matrix (ECM) proteins or its derivatives into alginate chains can compensate this deficiency and has attracted the interest of numerous scientists. Thus, several adhesion ligands have been employed to promote and regulate cellular interactions, providing the encapsulated cells with a microenvironment that mimics more closely their natural conditions [14-16]. The short-sequence peptide derived from fibronectin, arginine-glycine-asparte (RGD), has been widely used for this purpose with positive effects on the behavior of encapsulated cells [17-19].

Another key factor which has implied a great impact on cell-based therapies is the emergence of mesenchymal stem cells (MSCs) [20]. Maybe one of the most important advantages of MSCs in encapsulation technology is their immunomodulatory properties. It is reported that an inflammatory environment can stimulate MSCs to elicit high levels of immunosuppressive factors that directly and indirectly immunoregulate cytotoxic cells [21,22]. The release of those immunosuppressive molecules (IL-6, IL-10…) and several

growth factors (TGF-β, VEGF, PDGF or EGF) also stimulate endogenous repair mechanisms while maintaining immune homeostasis [23-25]. Another advantage is that MSCs can be passaged many times maintaining the multilineage differentiation potential into osteogenic, adipogenic and chondrogenic lineages due to their self-renewal capacity, [26]. In addition, they can be genetically modified in order to secrete bioactive molecules [27,28]. All this biological properties make this cell type a powerful tool for cell microencapsulation technology.

With the aim to move this therapy a step forward to clinical application, we genetically modified D1 Mesenchymal Stem Cells (D1-MSCs) to secrete erythropoietin (EPO) and encapsulated them into 3D biomimetic scaffolds. In previous studies our research group has described the influence of different RGD densities on fibroblasts releasing VEGF [29] and myoblasts releasing EPO [30], showing an enhancement of the therapeutic molecule delivery when cells were immobilized into RGD-modified alginate being the optimal RGD density different for each cell type. In this work, the viability, proliferation and EPO secretion of microencapsulated D1-MSCs was first evaluated *in vitro* and next these microcapsules were implanted in an allogenic animal model. Finally, the phenotypic and multilineage differentiation potential of the encapsulated cells was assessed *in vitro* and *in vivo*. To the best of our knowledge, this is the first report involving MSCs encapsulated in alginate modified with different RGD densities for drug delivery purposes.

2. Materials and methods

2.1. Cell culture and phenotypic analysis

D1 Mesenchymal Stem Cells (D1-MSCs) were purchased from ATCC (ATCC® CRL12424™). Cells were genetically modified with the lentiviral vector pSIN-EF2-Epo-Pur to express Erythropoietin (EPO) [31] and further grown and selected in Dulbecco´s modified Eagle´s medium (DMEM) (ATCC 30-2002) supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 12.5 µg/ml puromicine solution. Cells were plated in T-flasks, maintained at 37 ºC in a 5% CO_2/95% air atmosphere and passaged every 2-3 days. Reagents were purchased from Gibco (Life technologies, Spain).

For flow cytometric analysis, D1-MSCs were stained with FITC-conjugated murine monoclonal antibodies (mAb) against CD29, CD44 and SCA-I, PE-conjugated murine mAb against CD73 and CD105, and PerCP-conjugated murine mAb against CD45 (Serotec, Kidlington, United Kingdom). Briefly, $2x10^5$ cells were incubated for 30 minutes at 4°C with appropriate concentrations of monoclonal antibodies in PBS containing 2% fetal bovine serum. Cells were rinsed and resuspended in PBS. Flow cytometric acquisition was performed on a FACScalibur cytometer (BD Biosciences, San Jose, CA). Cells were primarily selected using forward and side scatter characteristics and fluorescence was analyzed using CellQuest software (BD Biosciences). Isotype-matched negative control antibodies were used in all the experiments. The mean relative fluorescence intensity was calculated by dividing the mean fluorescent intensity (MFI) by the MFI of its negative control.

2.2. Differentiation of D1-MSCs

The multipotency of D1-MSCs was determined by cell differentiation capacity into three lineages, osteoblasts, adipocytes and chondrocytes. Briefly, to induce osteogenic and adipogenic differentiation 10^5 cells were plated in 6 well culture plates until achievement of 70-80 % confluence. Osteogenic differentiation was induced by growth medium supplemented with Dexamethasone 0.1 μm, β-glycerophosphate 20 mM and L-ascorbic acid 50 μM. For adipogenic differentiation, growth medium was replaced by the medium supplemented with Dexamethasone 0.5 μM, Isobutylmethylxanthine 0.5 μM and Indomethacin 50 μM. To induce chondrogenic differentiation, D1-MSCs were resuspended at a density of $1.6x10^7$ cells/ml seeded in 3 droplets of 5 μl per well and incubated for 2 hours. Next media was replaced with growth media supplemented with 10 ng/ml TGF-β1, 50 nM L-ascorbic acid and 6.25 μg/ml bovine insulin. Each differentiation media was replaced every 2-3 days for 21 days. Finally, cells were fixed and stained with Alizarin Red S (osteogenic differentiation), Oil Red O (adipogenic differentiaiton) and Alcian Blue (chondrogenic differentiation). Reagents were purchased from Sigma-aldrich.

2.3. EPO secretion of differentiated D1-MSCs

The supernatants of differentiated and no differentiated cells were assayed in triplicate per study group for EPO secretion. Quantikine IVD Human Erythropoietin ELISA Kit purchased from R&D Systems (Minneapolis, MN) was employed for EPO secretion measurement. The results were normalized with the cell number in each well and expressed as mean ± S.D.

2.4. Incorporation of adhesion molecules into alginate

Alginate was chemically modified by the aqueous carbodiimide chemistry. A water soluble carbodiimide, (1-ethyl-dimethylaminopropyl) carbodiimide (EDC), was used to form amide linkages between amine containing molecules and the carboxylate moieties on the alginate polymer backbone, with a reaction efficiency of approximately 80% [32]. The total number of RGD peptides per alginate chain, defined as the degree of substitution (DS) [33], was altered by varying the concentration of RGD peptides in the coupling reaction, obtaining four different types of alginate: DS 0 (No modified alginate), DS 1 (0.112 mM), DS 5 (0.5 mM) and DS 10 (1.12 mM). Ultra pure low-viscosity high guluronic acid alginate (UPLVG) was purchased from FMC Biopolymer, Norway. RGD was achieved from Peptides International (Louisville, Kentucky, USA).

2.5. Cell microencapsulation

D1-MSCs genetically modified to release EPO were incorporated into 3D alginate-poly-l-lysine-alginate (APA) microcapsules using an electrostatic droplet generator with brief modifications of the procedure designed by Lim and Sun [6]. Briefly, cells were harvested from monolayer cultures using trypsin-EDTA (Life technologies), filtered through a 40 µm pore mesh and suspended in four different solutions of 1.5% (w/v) sodium alginate (DS 0, DS 1, DS 5, DS 10) at 10^7 cells/ml density. These suspensions were extruded in a sterile syringe through a 0.35 mm needle at a 5.9 mL/h flow rate using a peristaltic pump. The resulting alginate particles were collected in a 55mM $CaCl_2$ solution and maintained under agitation for 15 min after the end of the process to ensure complete gelation of all the beads. Then, beads were suspended in 0.05% PLL solution for 5 min, washed twice with 10 mL of manitol 1% and coated again with another layer of 0.1% alginate for 5 min. All the process was carried out under aseptic conditions at room temperature,

and resulting microcapsules were cultured in complete medium at 37 ºC in a 5% CO_2/95% air atmosphere standard incubator. Poly-l-lysine (PLL hidrobromide Mw 15 000–30 000 Da) was obtained from Sigma Aldrich.

2.6. Viability of encapsulated D1-MSCs

To assess the viability, cells entrapped into APA microcapsules were dyed with the LIVE/DEAD kit (Life technologies) following manufacturer's indications. After 30 min, fluorescence micrographs were taken using an epi-fluorescence microscope (Nikon TSM). To quantify the number of living cells, enclosed cells were firstly de-encapsulated with alginate lyase (0.5 mg/mL) (Sigma-Aldrich), and stained with LIVE/DEAD kit (Life technologies). After 20 min incubation at room temperature protected from light, cells were counted by flow cytometry (BD FACSCalibur) using Trucount Tubes (BD). All samples were assayed in triplicate for all groups and btained values are shown as mean of 3 independent samples ± S.D per study group. The number of living cells obtained for the day 0 was considered as 100% in each microcapsules group, and all values were expressed in function of this percentage.

2.7. Proliferation of encapsulated D1-MSCs

To assess proliferation of encapsulated D1-MSCs, the equivalent of 2×10^4 cells/100 µL (≈100 microcapsule/ well) was placed into each well of 96-well plate. All groups were incubated with complete medium supplemented with 10% FBS except the negative control group, which was incubated with starving medium supplemented with 0.1% FBS. After 24 h, the encapsulated cells were incubated in the presence of 10 µM BrdU for an additional day, except non-specific binding control group (without BrdU). The third day cells were de-encapsulated using alginate lyase (0.5 mg/ml) (Sigma-Aldrich) and assayed for BrdU uptake using Cell Proliferation Biotrak ELISA System (Amersham, NJ, USA) following manufacturer's indications. Absorbance measurements of the non-specific binding control group were subtracted from the rest of the groups, and results were normalized with the negative control (microcapsules incubated with 0.1 % FBS). Data are shown as mean of 5 independent samples ± S.D per study group. Results were normalized with those obtained with DS 0 group each day.

2.8. EPO secretion of encapsulated D1-

MSCs

In the case of encapsulated D1-MSCs, supernatants of all types of microcapsules were assayed for EPO secretion using the aforementioned kit. Standards and samples were run in duplicate according to the procedure specified in the kit. EPO secretion of the equivalent of 100 µl of microcapsules (10^7 cells/ml) was measured for a 24 h release period in triplicate per study group, and results are expressed as mean ± S.D.

2.9. Differentiation of encapsulated D1-MSCs

To induce adipogenic, osteogenic and chondrogenic differentiation of D1-MSCs within APA microcapsules, encapsulated cells were maintained in each conditioned media for 3 weeks. Next, capsules were stained with the specific staining in each case. In a second set of experiments, D1-MSCs maintained in their specific conditioned media to induce three types of differentiations were retrieved from the microcapsules using alginate lyase (0.5 mg/ml). The de-encapsulated cells were replated and stained according to the Alizarin Red and Oil Red O staining protocols. Alizarin Red staining was extracted with 6M HCl. Oil Red O staining was extracted with pure isopropanol. The degree of osteogenic and adipogenic differentiation was quantified by determining the absorbance of the extracts at 490 nm.

2.10. In vivo study: Microcapsule implantation, hematocrit measurement and microcapsule retrieval to evaluate explanted encapsulated cells

Animal studies were carried out according to the ethical guidelines established by our Institutions, under an approved animal protocol (CEEA/374/2014/HERNANDEZ MARTÍN). Adult female C57BL/6J mice were anesthetized by isoflurane inhalation, and implanted subcutaneously with a total volume of 300 µL of cell-loaded microcapsules ($10x10^6$ cells/mL) suspended in PBS using a 20-gauge catheter (Nipro; Nissho Corp, Belgium). Animals were housed in specific pathogen free facility under controlled temperature and humidity with a standardized 12 h light/dark cycle and had access to food and water ad libitum. Blood was collected before the administration and during 1 month at different time points (Day 7, 14, 21, 25 and 30) from the submandibular area using heparinized capillary tubes. Hematocrits

were determined after centrifugation at 3000 rpm for 10 min of whole blood using a standard microhematocrit method.

At day 15 and 30 after implantation, 3 animals from each group were sacrificed and capsules were explanted. Briefly, a mix of collagenase H (2mg/ml) and hyaluronidase (1mg/ml) (Sigma, St. Louis, USA) was prepared using DMEM. This enzyme solution was filtered-sterilized prior to use. Using 50 mL tubes, 5-6 mL of disgregation solution was added to each microcapsule aggregate and incubated in a shaker bath at 37 ºC for 4 h at 100 rpm. Once the surrounding tissue was disaggregated, the solution in the tubes was filtered using 40 µm pore size filters to recover tissue-free capsules. The aforementioned viability assay, EPO secretion quantification and phenotypic analysis were performed with the explanted microcapsules.

2.11. Statistical analysis

Data are presented as mean ± S.D. One-way ANOVA and post-hoc test were used in multiple comparisons. The Bonferroni or Tamhane post-hoc test was applied according to the result of the Levene test of homogeneity of variances. In the case of non-normally distributed data, Mann-Whitney non-parametric analysis was used. All statistical computations were performed using SPSS 20 (SPSS, Inc., Chicago, IL).

3. Results

3.1. Phenotypic analysis and differentiation of D1-MSCs

Cultured D1-MSCs were characterized by flow cytometry for standard mesenchymal stem surface markers. The histograms of the Figure 1A showed no expression of hematopoietic marker CD 45, but positive staining for MSC markers CD29, CD44, CD73, CD105 and SCA-I.

According to the tri-lineage differentiation capacity [34], D1-MSCs were differentiated into osteogenic, adipogenic and chondrogenic lineages. Detection of calcium deposits by Alizarin Red, the cytoplasmatic accumulation of vacuoles filled with neutral lipids by Oil Red O and the positive Alcian blue staining for cartilage matrix confirmed the differentiation potential of evaluated cells (Fig. 1B).

3.2. EPO secretion of differentiated D1-MSCs

EPO secretion of differentiated and

no differentiated cells were measured to observe differences between the different cell phenotypes (Fig. 1C). EPO secretion showed statistical differences between chondrogenic differentiated and control cells at the second week (p<0.05), while at the third week the differences were between chondrogenic and osteogenic lineages (p<0.05). In both cases chondrogenic lineage showed the lowest EPO values, while undifferentiated MSCs and osteogenic lineage cell showed the highest EPO secretion. Based on these results, undifferentiated D1-MSCs were selected to follow up with experiments of encapsulated cells.

3.3. Viability of encapsulated D1-MSCs

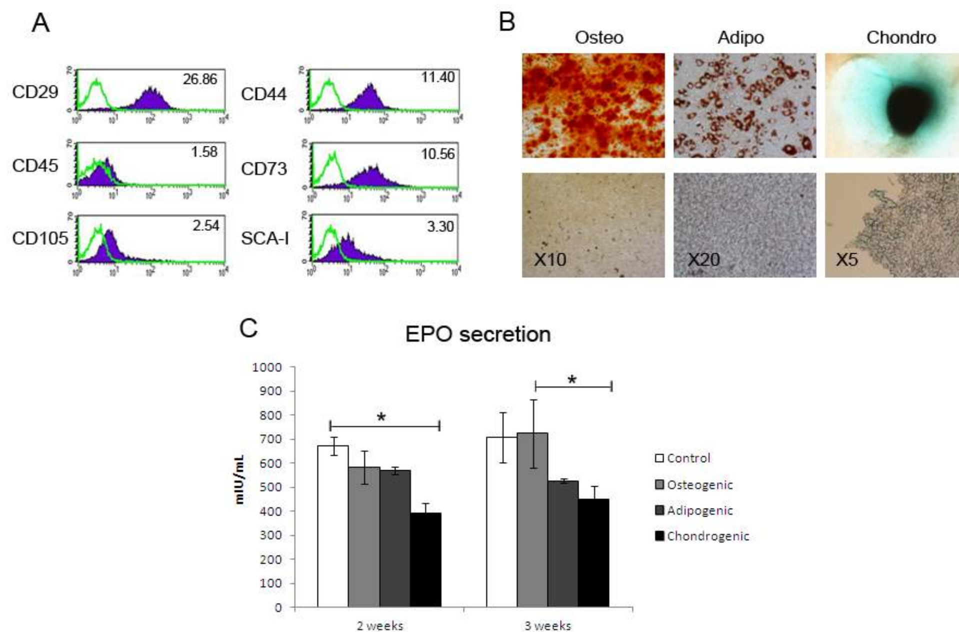


Figure 1. Phenotypic analysis of D1-MSCs in culture by FACS. Representative graphs of cell markers expression (filled histograms) and their isotype control (green lined histograms) are shown. Values in the graphs indicate the expression level represented as Mean Relative Fluorescence Intensity (MRFI), calculated by dividing the Mean Fluorescence Intensity (MFI) by its negative control (A). Photomicrographs showing the differentiation potential of D1-MSCs. Osteogenic, adipogenic and chondrogenic differentiation with their respective negative controls on the second row (B). EPO secretion of D1-MSCs cultured in specific media to induce their differentiation. Bar graphs symbolize the mean ± S.D (n=3). Statistical significance *p < 0.05 (C).

Once D1-MSCs were characterized based on the expression of MSC markers, and the tri-lineage differentiation capacity, no differentiated D1-MSCs were encapsulated within alginate microcapsules modified with different RGD densities (Fig. 2). The viability of all types of microcapsules with spherical morphology and a homogeneous size distribution (diameter 450-470 µm) was evaluated during 21 days. Fluorescence micrographs collected by day 21 demonstrated the high viability of cells encapsulated in all types of microcapsules maintaining the green fluorescence of living cells even at the end of the study (Fig. 3A). Data obtained by flow cytometry provided further evidence on our observations. Although at day 15 slight intergroup differences were observed among cells encapsulated with the highest RGD densities and cells immobilized in microcapsules without RGD, at day 21 all the viabilities were similar, maintaining a high level of living cells (75-80 %) (Fig. 3B).

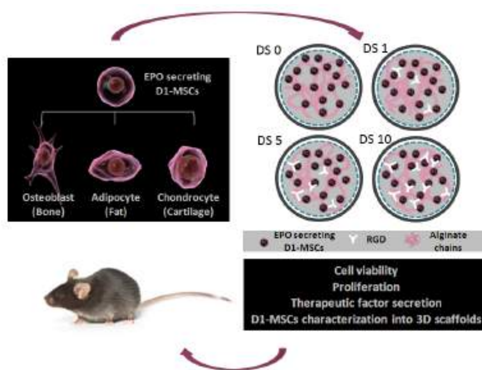


Figure 2. Schematic illustration of the main objectives of the study: First, the characterization of EPO secreting D1-MSCs, including their differentiation potential. Second, the immobilization of no differentiated cells into alginate microcapsules with different RGD densities to its *in vitro* analsysis. Finally, the implantation of microcapsules into mice to evaluate cell behavior *in vivo*.

3.4. Proliferation and EPO secretion of encapsulated D1-MSCs

Proliferation of D1-MSCs encapsulated in biomimetic scaffolds was evaluated to determine whether inclusion of adhesion moieties could enhance DNA synthesis of cells. As shown in the Figure 3C, the alginate modification had a significant effect on the D1-MSCs proliferation increasing significant intergroup differences over time. At day 15, DS 1 and DS 5 groups showed higher BrdU uptake compared with DS 0 and DS 10 groups. At the end of the study, the differences were more evident obtaining almost 1.5 fold higher BrdU uptake in the DS 1 and DS 10 groups ($p<0.01$), and an 1.85 ± 0.11-fold enhancement of DNA synthesis in the DS 5 group ($p<0.001$). These data

come along with the results obtained in the therapeutic factor secretion. In fact, from the beginning of the study, D1-MSCs encapsulated in RGD-modified alginates showed a higher activity obtaining an enhancement in the EPO secretion (Fig. 3D). Although differences were statistically significant in all groups with RGD at day 1, at day 15 only the groups with intermediate levels of the tripeptide (DS 1 and DS 5) maintained statistical differences (p<0.01, p<0.05). By day 30, DS 5 was the unique group with statistically significant enhancement of EPO secretion compared with DS 0 group, achieving values of 2855 ± 266 mIU/ml (p<0.05).

3.5. Phenotypic analysis and capacity of differentiation potential of encapsulated D1-MSCs

The phenotype of encapsulated D1-MSCs was analyzed by flow cytometry. The results showed that D1-MSCs immobilized in alginate matrices modified

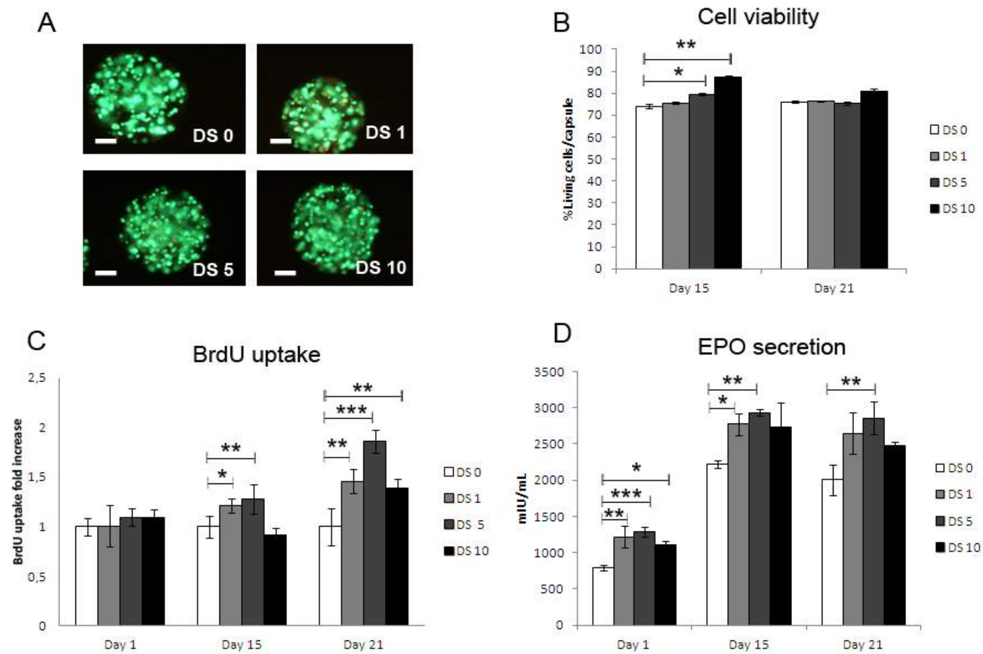


Figure 3. Fluorescence micrographs of cells encapsulated into different alginate matrices at day 21 (A). Percentage of living cells after 15 and 21 days of encapsulation (B), BrdU uptake (C) and EPO secretion (D) at day 1, 15 and 21 of the study. Bar graphs symbolize the mean ± S.D . Statistical significance *p < 0.05, **p < 0.01 and ***p < 0.001 compared with DS 0 group. Scale bars = 100 µm.

with different RGD densities maintained their stem cells phenotype. As shown in the Figure 4A, cells encapsulated in all types of microcapsules had no expression of CD 45 while positively expressed the MSC markers CD29, CD44, CD73, CD105 and SCA-I without significant differences between groups by day 21.

The encapsulated D1-MSCs were evaluated for their ability to differentiate into the 3 mesoderm lineages (osteoblasts, adipocytes and chondrocytes) within the microcapsules and after retrieval. The

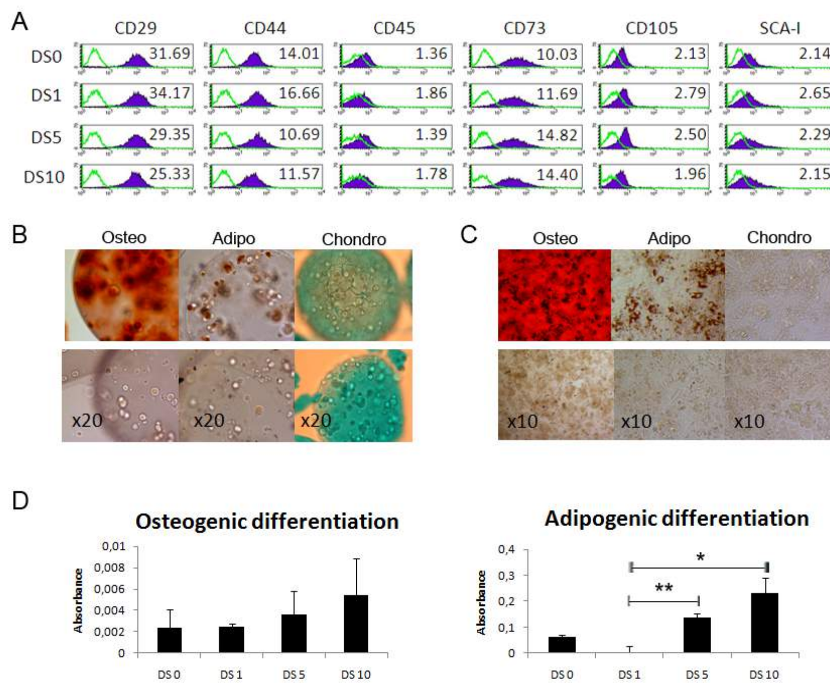


Figure 4. Phenotypic characterization of de-encapsulated D1-MSCs from all type of alginate matrices by FACS. Representative graphs of cell markers expression (filled histograms) and their isotype control (green lined histograms) are shown. Values in the graphs indicate the expression level represented as Mean Relative Fluorescence Intensity (MRFI), calculated by dividing the Mean Fluorescence Intensity (MFI) by its negative control (A). Diferentiation potential of microencapsulated D1-MSCs cultured 21 days in conditioned media. Osteogenic, adipogenic and chondrogenic differentiation with their respective negative controls within microcapsules (B) and after de-encapsulation (C). Quantification of osteogenic and adipogenic differentiation quantified by determining the absorbance of the extracts at 490 nm (D). Bar graphs symbolize the mean ± S.D. Statistical significance *$p < 0.05$ and **$p < 0.01$.

deposition of mineralized bone matrix and lipid vacuoles of adipocytes were identified by the previous mentioned staining not only in the microcapsules but also after de-encapsulation of D1-MSCs (Fig. 4B and 4C). However, we could not determine differentiation into chondrocytes, since the negative control also was stained with Alcian Blue (Fig. 4B). Moreover, when cells were stained after retrieval from the capsules, there were no differences between the chondrogenic and control group (Figure 4C). The absorbance values of the extracts obtained from different stains demonstrated that RGD-modified microcapsules tend to enhance the differentiation potential of D1-MSCs (Figure 4D). Although there were not differences between DS 0 and other groups, the microcapsules elaborated with the highest RGD densities showed the highest absorbance values obtaining statistical differences compared to DS 1 group (p<0.01, P<0.05).

3.6. Hematocrit levels after subcutaneous administration of microcapsules

In a second set of experiments, all types of microcapsules that were previously evaluated *in vitro* were implanted subcutaneously in mice to observe the effects of RGD *in vivo*. At day 15 and 30, the microcapsules were retrieved to further evaluate the immobilized cells. During all the study, hematocrit levels of mice were recorded at different time points (Fig. 5A). Hematocrit levels showed that all groups underwent an equal increase during all the experiment. No significant differences were observed among the different groups, obtaining the highest hematocrit values at day 25, with a hematocrit level around 90% in all cases (Fig. 5B).

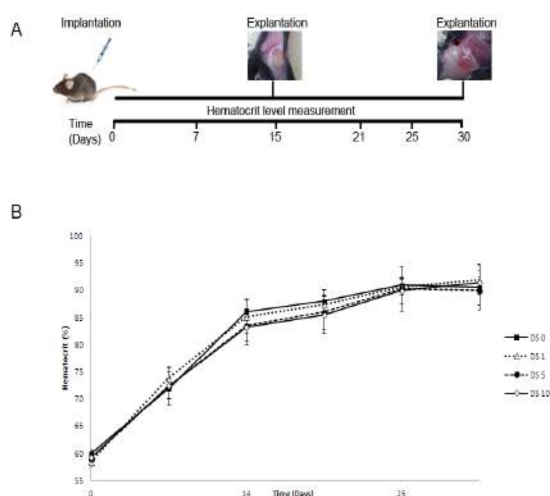


Figure 5. Schematic illustration of the *in vivo* study showing different time points for microcapsule explantation and hematocrit level mesurements (A). Hematocrit levels of C57BL/6J mice after subcutaneous implantation of EPO secreting D1-MSCs immobilized in different matrices (DS 0, DS 1, DS 5 and DS 10) (B).

3.7. Encapsulated cell viability, EPO secretion and phenotypic analysis in vivo

As shown in Figure 6A, cell viability was similar among all groups at day 15. EPO secretion also was similar in all groups (Fig. 6B), achieving values around 1500 mIU/ml, lower values than those achieved in the *in vitro* assay by the same day. The stem cell phenotype of MSCs was analyzed after retrieval of implanted microcapsules at day 15 and 30 (Fig. 6C and 7C). The results showed that phenotype of MSCs ($CD29^{+}CD44^{+}CD45^{-}CD73^{+}CD105^{+}SCA\text{-}I^{+}$) is preserved in all conditions assayed with a similar phenotype profiles than D1-MSCs cultured in different types of microcapsules *in vitro*.

Although at day 30 encapsulated cells maintained a high viability, the photographs also highlighted the

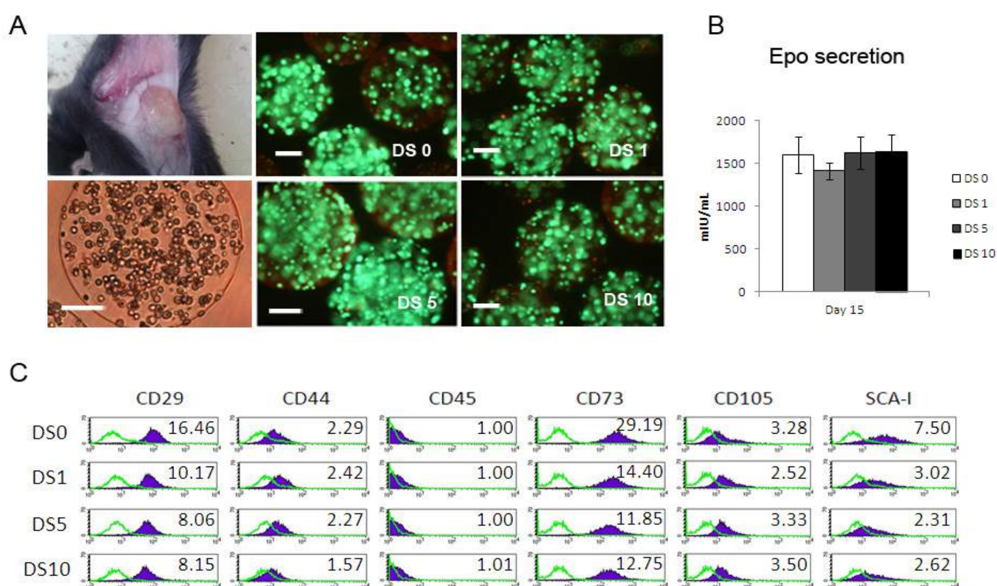


Figure 6. Microcapsules explanted at day 15: morphology of D1-MSCs and fluorescence micrographs of cells encapsulated into different alginate matrices at day 15 after implantation (A). EPO secreted of encapsulated cells after retrieval of mice at day 15 (B) Phenotypic characterization of D1-MSCs de-encapsulated of all type of alginate matrices at day 15 by FACS. Representative graphs of cell markers expression (filled histograms) and their isotype control (green lined histograms) are shown. Values in the graphs indicate the expression level represented as Mean Relative Fluorescence Intensity (MRFI), calculated by dividing the Mean Fluorescence Intensity (MFI) by its negative control (C).

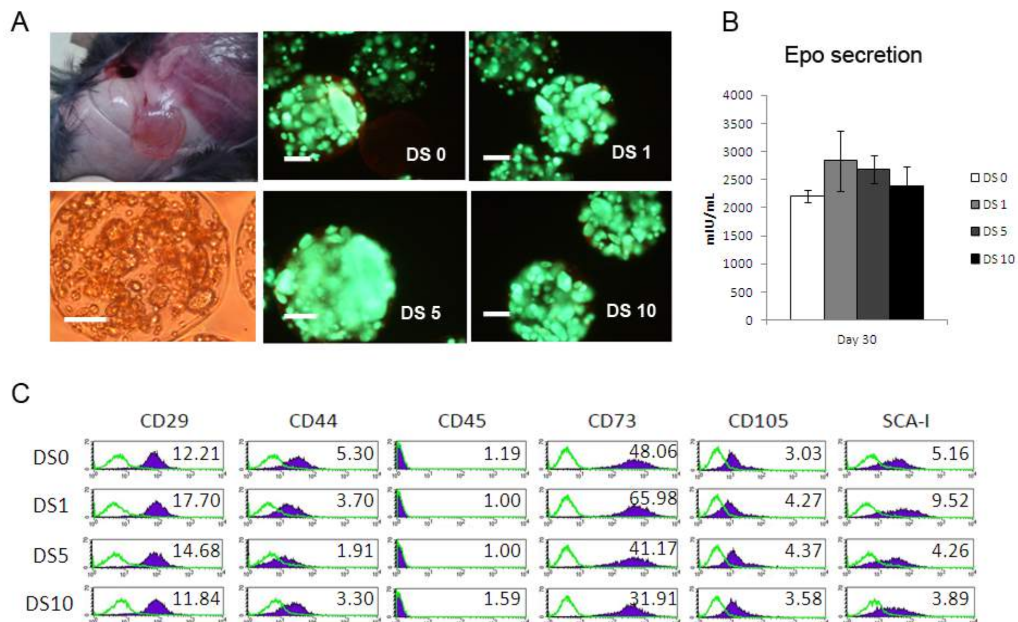


Figure 7. Microcapsules explanted at day 30: morphology of D1-MSCs and fluorescence micrographs of cells encapsulated into different alginate matrices taken by day 30 (A). EPO secreted of encapsulated cells after retrieval of mice at day 30 (B) Phenotypic characterization of D1-MSCs de-encapsulated of all type of alginate matrices at day 30 by FACS. Representative graphs of cell markers expression (filled histograms) and their isotype control (green lined histograms) are shown. Values in the graphs indicate the expression level represented as Mean Relative Fluorescence Intensity (MRFI), calculated by dividing the Mean Fluorescence Intensity (MFI) by its negative control (C).

apparition of some cell aggregates (Fig. 7A). There were no intergroup statistical differences in the EPO secretion at the end of the study but the values obtained at day 30 were higher than those obtained at day 15 in the *in vivo* experiment (Fig. 7B).

4. Discussion

The immobilization of diverse cell types into 3D scaffolds represents an attractive tool to enable the local and controlled delivery of therapeutic molecules. However, despite the design of alginate-poly-l-lysine-alginate (APA) microcapsules protecting the implanted cells from the

host immune response, the encapsulated nonautologous cells secrete cytokines and shed antigens. These factors eventually evoke a host immune response giving rise to an inflammatory tissue surrounding the microcapsules that leads to suffocation and death of the encapsulated cells [35,36]. The use of MSCs that can downregulate or reduce this immune response has involved a great advance on the field, offering promising properties for cell microencapsulation and cell-based therapies [37,38]. In addition, these cells can be also genetically modified to achieve a secretion of the desired therapeutic factor depending on the intended application.

Besides the importance of a judicious choice of cells for the efficacy of this therapeutic strategy, the success or failure of cell microencapsulation systems also depends on the properties of the scaffold where cells are immobilized [39]. Focusing on that issue, one of the major design strategies is the modification of inert polymeric matrices with bioactive molecules in order to provide cells with a more natural environment [11]. Among others, small amino acid sequences as RGD have been employed as cell recognition motives in inert scaffolds such as alginate matrices [40]. After the promising results obtained in previous works encapsulating C_2C_{12} [30] and BHK cells [29] in RGD-modified alginates, the present work evaluates the properties of EPO secreting D1-MSCs into different microcapsules in order to make a step forward in cell encapsulation technology. Thus, we have provided interesting information regarding the effect of different RGD densities on encapsulated D1-MSCs *in vitro* and *in vivo*.

The phenotypic analysis and the multi-lineage differentiation potential revealed that stem cell characteristics of genetically modified D1-MSCs remained intact (Fig. 1 A-B). Due to the possibility to observe a variation in the EPO expressed from different lineages of D1-MSCs, the therapeutic factor secretion from differentiated osteoblasts, adipocytes and chondrocytes was evaluated (Fig. 1C). The results demonstrated that undifferentiated and cells differentiated into osteogenic and adipogenic lineages maintained similar EPO expression while the chondrogenic lineage achieved lower levels. This difference may be attributable to the specific culture conditions to induce a chondrogenic differentiation which vary significantly from the osteo-

and adipo-lineages induction [28]. For the differentiation to chondrogenic lineage, D1-MSCs should be cultured in a dense 3D environment, which can lead to a reduction of cell viability. In addition, the secreted EPO could be retained by the dense tissue environment hindering its diffusion to the cell culture media. Despite the therapeutic factors decrease showed by the chondrogenic lineage, D1-MSCs maintained a sustained expression of EPO following differentiation, a very desirable feature for drug delivery systems.

D1-MSCs maintained a high viability in 3D alginate scaffolds (75-80%) during 21 days with slight differences between microcapsules elaborated with or without RGD. This could be explained by the fact that while some cell types need interactions for the attachment of the inner core, cell anchorage is not a strict requirement for survival of D1-MSCs. Nevertheless, the results shown in the Figure 3C and 3D revealed that RGD added to the alginate matrices plays critical roles in some important cellular functions. According to the results collected in other works, the inclusion of RGD leads to an enhancement of the proliferation of encapsulated cells. It is noteworthy that low and intermediate levels of RGD (DS 1 and DS 5) were the most effective to obtain the highest proliferation activity [41]. This is consistent with previous studies that also reported the inhibitory effect of RGD when too high densities are employed for surface modifications [42]. In accordance with the proliferation assay, EPO secretion is also enhanced in the case of D1-MSCs immobilized into microcapsules modified with RGD, obtaining the highest values with the DS 5 group. It should be noted that EPO secretion increases over time in all conditions, regardless of the RGD modification. The constant viability values obtained during all the experiment lead to the hypothesis that the enhancement of EPO released from D1-MSCs can be related with a higher activity of enclosed cells.

The differentiation potential of encapsulated cells upon specific induction was confirmed by the presence of calcium deposits (osteogenic lineage) and lipid vacuoles (adipogenic lineage) (Fig. 4B and 4C). These differentiations occurred in all types of microcapsules (data not shown), but slight differences could be observed after the absorbance measurement of the extracts obtained from de-encapsulated cells (Fig. 4D). Our findings come along with other studies which reported that

apart from its influence on different cell functions, RGD also promotes the differentiation of MSCs immobilized into 3D scaffolds [43,44].

Interestingly, our RGD-modified alginate matrices did not provide a suitable environment for chondrogenic differentiation of D1-MSCs. Previous studies showed that cell aggregates are needed to induce chondrogenic differentiation in MSCs in 3D cultures [38,45], and the solid core of APA microcapsules does not facilitate that process. When the polymeric matrices have high restriction forces as in the case of our microcapsules, the cells normally proliferate based on the local cells and grow into small multispheroids hindering the chodrogenic differentiation [12].

In a second set of experiments, we moved on to an *in vivo* assay to evaluate the behavior of D1-MSCs entrapped in different scaffolds in an allogenic recipient. The elevated and similar hematocrit level obtained by all groups suggests that the fact of adding RGD into alginate does not have a significant effect on the EPO secretion (Fig. 5). To provide a more consistent data regarding that issue, EPO released by cells was analzyzed after the explantation of microcapsules from the animals. Obtained data demonstrated that in fact, there were no differences between EPO secreted by D1-MSCs immobilized in different types of microcapsules (Fig. 6B and 7B). However, it is important to note that although EPO levels were lower than those obtained in the *in vitro* assay, the implanted D1-MSCs also increase EPO secretion over time in all *in vivo* conditions. The aggregates formed by these cells into all types of microcapsules at day 30 (Fig. 7A) give an explanation to the enhancement of the therapeutic factor secretion along the 30 days period of the study [46]. Nevertheless, although the EPO released from all microcapsules explanted at day 15 was very similar, at the end of the study some differences could be detected between the different groups, obtaining the highest levels in the microcapsules with low and intermediate densities of RGD (DS 1 and DS 5).

The simultaneous analysis of a wide range of stem cell-related markers demonstrated that D1-MSCs immobilized into all microcapsules and cultured in basal media without differentiation induction were similar in terms of surface markers expression (Fig. 4A). According to the phenotypic analysis, it can be

assumed that encapsulation of these cells into different matrices did not induce the differentiation towards adipo-, osteo- and chondrogenic lineages. Indeed, although the differentiation process may occur *in vivo* under certain circumstances [26], our *in vivo* experiments confirmed that MSCs remained in an undifferentiated state maintaining their self-renewal potential (Fig. 6C and 7C).

The results collected in the present study indicate that the inclusion of RGD into alginate matrices do not affect the behavior of encapsulated cells *in vivo* as much as *in vitro* studies during the period of 30 days. These differences may be attributable to the diverse growth factors and hormones surrounding microcapsules environment *in vivo* that can alter vital cellular functions of D1-MSCs. The aforementioned aggregates D1-MSCs observed in the implanted microcaspules revealed that cell proliferation increases even into alginate without RGD. This suggests that the complexity provided by the *in vivo* body fluids to the microcapsules environment may have more influence than the adhesion moieties into the matrices for this cell type. However, it should be highlighted that a longer *in vivo* follow-up period of the implanted microcapsules could allow observing more differences between different groups [19].

5. Conclusions

This study shows that genetically modified EPO secreting D1-MSCs present appropriate characteristics to be employed as a vehicle for drug delivery systems, since the inclusion of these cells into alginate microcapsules do not affect their main properties. The use of RGD as bioactive molecule involves a higher activity of encapsulated D1-MSCs, not only increasing therapeutic factor secretion, but also enhancing the differentiation potential of immobilized cells upon specific induction. Finally, the present work shows that subcutaneous implantation of microcapsules does not induce differentiation of D1-MSCs towards any lineage remaining at an undifferentiated state *in vivo*.

Acknowledgements

Authors would like to thank Dr Felipe Prosper at the Clínica Universidad de Navarra (CUN) for his assistance on the development of the lentiviral vector pSIN-

EF2-Epo-Pur. The authors also gratefully acknowledge the support to research cell microencapsulation from the "Ministerio de Ciencia e Innovación", University of the Basque Country UPV/EHU (UFI11/32). Authors also wish to thank the intellectual and technical assistance from the platform for drug formulation, CIBER-BNN. A. Garate thanks the "Gobierno Vasco (Departamento de Educación, Universidades e Investigación)" for the Ph.D fellowship.

References

1 Acarregui A, Murua A, Pedraz JL, Orive G, Hernandez RM. A perspective on bioactive cell microencapsulation. BioDrugs 26, 283-301 (2012).

2 Saenz Del Burgo L, Compte M, Aceves M, et al. Microencapsulation of therapeutic bispecific antibodies producing cells: immunotherapeutic organoids for cancer management. J.Drug Target. 23, 170-179 (2015).

3 Safley SA, Cui H, Cauffiel SM, Xu BY, Wright JR,Jr, Weber CJ. Encapsulated piscine (tilapia) islets for diabetes therapy: studies in diabetic NOD and NOD-SCID mice. Xenotransplantation 21, 127-139 (2014).

4 Nafea EH, Marson A, Poole-Warren LA, Martens PJ. Immunoisolating semi-permeable membranes for cell encapsulation: focus on hydrogels. J.Control.Release 154, 110-122 (2011).

5 Hernandez RM, Orive G, Murua A, Pedraz JL. Microcapsules and microcarriers for in situ cell delivery. Adv.Drug Deliv.Rev. 62, 711-730 (2010).

6 Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 210, 908-910 (1980).

7 Gurruchaga H, Saenz Del Burgo L, Ciriza J, Orive G, Hernandez RM, Pedraz JL. Advances in cell encapsulation technology and its application in drug delivery. Expert Opin.Drug Deliv. 1-17 (2015).

8 Santos E, Pedraz JL, Hernandez RM, Orive G. Therapeutic cell encapsulation: ten steps towards clinical translation. J.Control.Release 170, 1-14 (2013).

9 Gasperini L, Mano JF, Reis RL. Natural polymers for the microencapsulation of cells. J.R.Soc.Interface 11, 20140817 (2014).

10 Shin H, Jo S, Mikos AG. Biomimetic materials for tissue engineering. Biomaterials 24, 4353-4364 (2003).

11 Santos E, Hernandez RM, Pedraz JL, Orive G. Novel advances in the design of three-dimensional bio-scaffolds to control cell fate: translation from 2D to 3D. Trends Biotechnol. 30, 331-341 (2012).

12 Huang X, Zhang X, Wang X, Wang C, Tang B. Microenvironment of alginate-based microcapsules for cell culture and tissue engineering. J.Biosci.Bioeng. 114, 1-8 (2012).

13 Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog.Polym.Sci. 37, 106-126 (2012).

14 Li C, Zhao S, Zhao Y, Qian Y, Li J, Yin Y. Chemically crosslinked alginate porous microcarriers modified with bioactive molecule for expansion of human hepatocellular carcinoma cells. J.Biomed.Mater.Res.B.Appl. Biomater. 102, 1648-1658 (2014).

15 Mazzitelli S, Luca G, Mancuso F, et al. Production and characterization of engineered alginate-based microparticles containing ECM powder for cell/tissue engineering applications. Acta Biomater. 7, 1050-1062 (2011).

16 Seo SJ, Akaike T, Choi YJ, Shirakawa M, Kang IK, Cho CS. Alginate microcapsules prepared with xyloglucan as a synthetic extracellular matrix for hepatocyte attachment. Biomaterials 26, 3607-3615 (2005).

17 Sayyar B, Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Cell-matrix Interactions of Factor IX (FIX)-engineered human mesenchymal stromal cells encapsulated in RGD-alginate vs. Fibrinogen-alginate microcapsules. Artif.Cells Nanomed Biotechnol. (2013).

18 Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. J.Biomed.Mater.Res. 60, 217-223 (2002).

19 Orive G, De Castro M, Kong HJ, et al. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. J.Control.Release 135, 203-210 (2009).

20 Attia N, Santos E, Abdelmouty H, et al. Behaviour and ultrastructure of human bone marrow-derived mesenchymal stem cells immobilised in alginate-poly-l-lysine-alginate microcapsules. J.Microencapsul. 31, 579-589 (2014).

21 Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood 105, 2214-2219 (2005).

22 Casado JG, Tarazona R, Sanchez-Margallo FM. NK and MSCs crosstalk: the sense of immunomodulation and their sensitivity. Stem Cell.Rev. 9, 184-189 (2013).

23 Boomsma RA, Geenen DL. Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. PLoS One 7, e35685 (2012).

24 Chen PM, Yen ML, Liu KJ, Sytwu HK, Yen BL. Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells. J.Biomed.Sci. 18, 49-0127-18-49 (2011).

25 Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. Cell Death Differ. 21, 216-225 (2014).

26 Juffroy O, Noel D, Delanoye A, Viltart O, Wolowczuk I, Verwaerde C. Subcutaneous graft of D1 mouse mesenchymal stem cells leads to the formation of a bone-like structure. Differentiation 78, 223-231 (2009).

27 McMahon JM, Conroy S, Lyons M, et al. Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral

vectors. Stem Cells Dev. 15, 87-96 (2006).

28 Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Sustained expression of coagulation factor IX by modified cord blood-derived mesenchymal stromal cells. J.Gene Med. 16, 131-142 (2014).

29 Garate A, Santos E, Pedraz JL, Hernandez RM, Orive G. Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems. J.Drug Target. 1-7 (2015).

30 Santos E, Garate A, Pedraz JL, Orive G, Hernandez RM. The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells: *In vitro* and *in vivo* direct comparative study. J.Biomed.Mater. Res.A. (2013).

31 Gurruchaga H, Ciriza J, Saenz Del Burgo L, et al. Cryopreservation of microencapsulated murine mesenchymal stem cells genetically engineered to secrete erythropoietin. Int.J.Pharm. 485, 15-24 (2015).

32 Rowley JA, Madlambayan G, Mooney DJ. Alginate hydrogels as synthetic extracellular matrix materials. Biomaterials 20, 45-53 (1999).

33 Comisar WA, Hsiong SX, Kong HJ, Mooney DJ, Linderman JJ. Multi-scale modeling to predict ligand presentation within RGD nanopatterned hydrogels. Biomaterials 27, 2322-2329 (2006).

34 Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315-317 (2006).

35 Orive G, Tam SK, Pedraz JL, Halle JP. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. Biomaterials 27, 3691-3700 (2006).

36 de Groot M, Schuurs TA, van Schilfgaarde R. Causes of limited survival of microencapsulated pancreatic islet grafts. J.Surg.Res. 121, 141-150 (2004).

37 Liu H, Lu K, Macary PA, et al. Soluble molecules are key in maintaining the immunomodulatory activity of murine mesenchymal stromal cells. J.Cell.Sci. 125, 200-208 (2012).

38 Goren A, Dahan N, Goren E, Baruch L, Machluf M. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. FASEB J. 24, 22-31 (2010).

39 Mallett AG, Korbutt GS. Alginate modification improves long-term survival and function of transplanted encapsulated islets. Tissue Eng.Part A. 15, 1301-1309 (2009).

40 Ruoslahti E. RGD and other recognition sequences for integrins. Annu.Rev.Cell Dev. Biol. 12, 697-715 (1996).

41 Neff JA, Tresco PA, Caldwell KD. Surface modification for controlled studies of cell-ligand interactions. Biomaterials 20, 2377-2393 (1999).

42 Comisar WA, Kazmers NH, Mooney DJ, Linderman JJ. Engineering RGD nanopatterned hydrogels to control preosteoblast behavior: a combined computational and experimental approach. Biomaterials 28, 4409-4417 (2007).

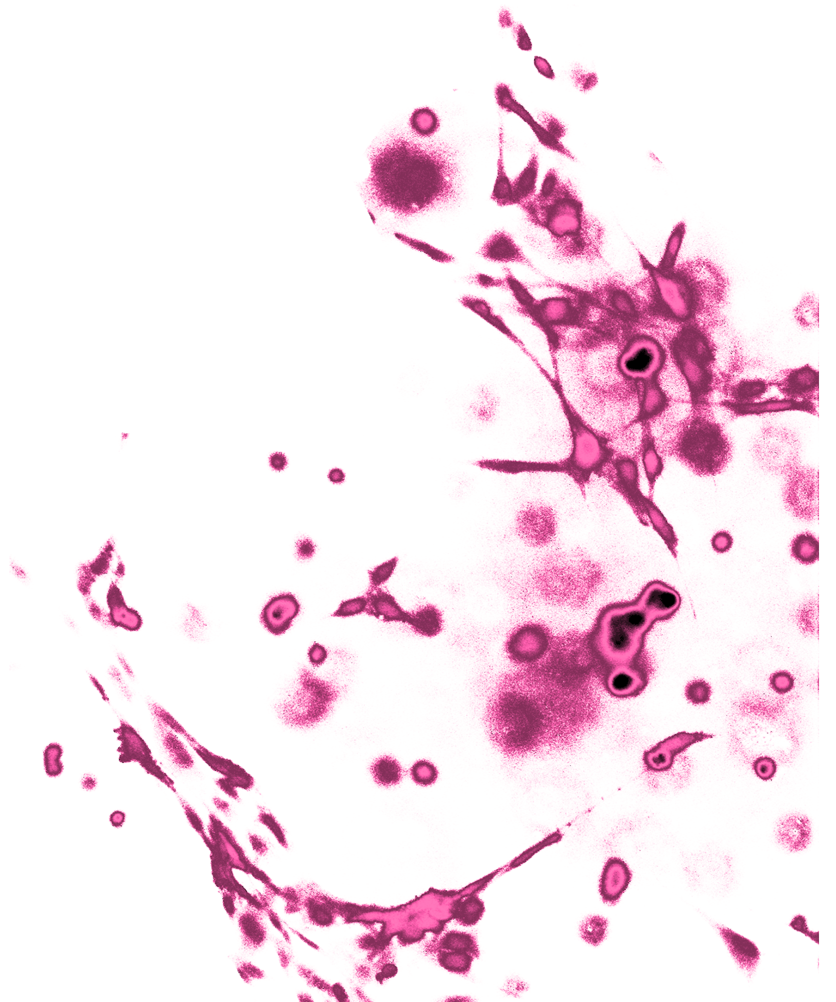
43 Evangelista MB, Hsiong SX, Fernandes R, et al. Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. Biomaterials 28, 3644-3655 (2007).

44 Grellier M, Granja PL, Fricain JC, et al. The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on mineralization in a bone defect. Biomaterials 30, 3271-3278 (2009).

45 Sayyar B, Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Fibronectin-Alginate microcapsules improve cell viability and protein secretion of encapsulated Factor IX-engineered human mesenchymal stromal cells. Artif.Cells Nanomed Biotechnol. (2014).

46 Jiang LY, Lv B, Luo Y. The effects of an RGD-PAMAM dendrimer conjugate in 3D spheroid culture on cell proliferation, expression and aggregation. Biomaterials 34, 2665-2673 (2013).

Discussion



Cell microencapsulation biotechnology represents a promising therapy to allow the immunoisolated transplantation of non-autologous cells. Since the first approach carried out more than 30 years ago several strategies have been developed leading to an evolution of this therapeutic strategy. However, the implementation of biosafety and monitorization systems to exert a tight control over the implanted cells, the improvement of biocompatibility as well as the reduction of the inflammatory responses are still pending issues which hinder the progress of this technology towards clinical reality. The preservation of cells with high viability in polymeric matrices is another unresolved challenge yet and new possibilities of biomaterial science are emerging to overcome this drawback.

Regarding this aspect, several research groups are working in the modification of polymeric matrices where cells are immobilized with bioactive molecules derived from extracellular matrix (ECM) which provide attachment to immobilized cells improving the functionality of cell-based 3D systems. The tripeptide arginine-glycine-aspartate (RGD) derived from longer ECM proteins, is one of the most employed adhesion molecule with this aim on the field, and many positive results are collected in the literature which support its potential as bioactive molecule. Nevertheless, while *in vitro* studies have confirmed the effectiveness of RGD peptides, *in vivo* studies have been shown to be more variable, opening an extended debate about its use. This doctoral thesis is intended to analyze some factors that may vary the effect of RGD in alginate microcapsules. Thus, the aim of the present studies has been to analyze the influence of RGD moieties on several cell types enclosed in 3D alginate capsules, trying to answer the following questions:

Is the choice of RGD density of relevant importance in the design of functional drug delivery system?

Is there an optimal RGD density that provides the highest activity for all cell types?

Are the results obtained in the *in vitro* experiments comparable with those obtained in an *in vivo* microenvironment?

Do D1-MSCs (Mesenchymal stem cells) maintain their stem cell properties when they are immobilized within RGD modified alginate scaffolds?

1. RGD density: Its influence on VEGF releasing BHK fibroblasts *in vitro*.

A broad range of cell lines has been enclosed within polymeric matrices surrounded by a semipermeable membrane designed to immune-protect the cell content from both mechanical stress and host's cellular immune rejection [1-5]. Alginate is a natural biomaterial widely used in cell encapsulation due to its several favorable properties, but it does not provide sufficient cues for cell-matrix interactions, being an inert polymer [6,7]. One way to prolong cell survival in these non-cell-interactive matrices is to modify them with the amino acid sequence arginine-glicine-aspartate (RGD), a short adhesion peptide found in fibronectin and other natural components of the ECM. The use of this tripeptide offers some advantages over the use of the whole protein; for example, simplicity, cost effectiveness, easy manipulation for functionalization and low immune response [8,9]. For these reasons, RGD has generated attention as a potential means to provide inert polymers with biological cues and thus extending the long term viability of the immobilized cells [10-12].

Although there is a significant biological foundation to support the activity of RGD moiety and an exhaustive literature has established that it is highly effective at promoting the attachment of numerous cell types in diverse biomaterials [13-17], some inconsistencies collected in recent works, especially the variable results obtained *in vivo*, have led to questioning its actual effectiveness. Indeed, the variability that offers this adhesion ligand might become a disadvantage in certain cases, which has generated some discrepancies in the scientific community to date [18-22]. Among others, the use of different RGD ligand types, the differences in RGD presentation patterns or the effect of the microenvironment enhancing/decreasing its activity are variables that must be taken into account when using RGD [23-26]. In this regard, the influence of adhesion ligand density is one of the main parameters that must be defined for each particular cell and application, being pivotal to obtain the optimum functionality of immobilized cells [21,27].

In the first work of this doctoral thesis, we aimed to evaluate the influence of RGD density on VEGF secreting BHK fibroblasts *in vitro*, encapsulating the cells in 4 different alginate matrices modified with increasing RGD densities: DS 0, DS 1, DS 5 and DS 10 (Fig. 1).

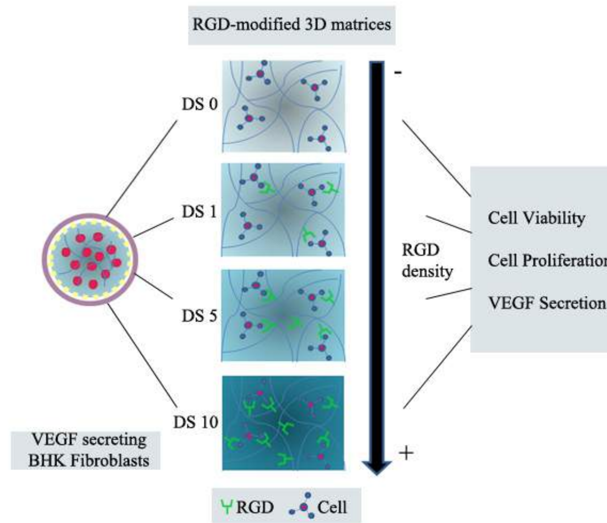
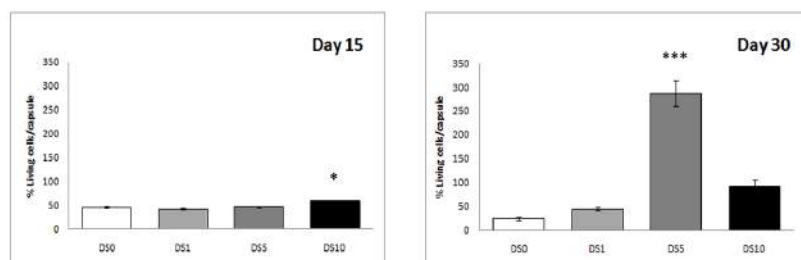


Figure 1. Schematic illustration of VEGF-secreting BHK fibroblasts encapsulated in 4 alginate matrices elaborated with different RGD densities.

The results obtained after the evaluation of viability, VEGF secretion and proliferation of fibroblasts encapsulated in different matrices clarified that intermediate levels of RGD (DS 5) provide the maximum functionality to the system (Fig. 2). Interestingly, the collected results demonstrated that too high RGD densities (DS 10) might produce an inhibitory effect. This phenomenon was explained by assuming that the strong adhesions resulting from many bound receptors may impede cell division, producing an inhibitory effect when the employed densities of RGD are too high [28].



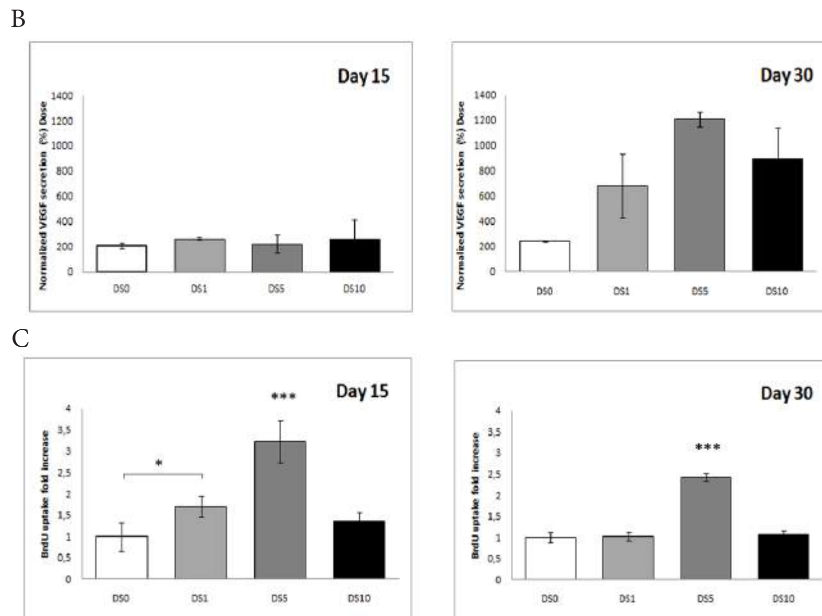


Figure 2. Percentage of living cells (A) and VEGF secretion (B) after 15 and 30 days of encapsulation. The number of living cells or VEGF secretion obtained for the day 0 was considered as 100% in each microcapsules group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). BrdU uptake (C) after 15 and 30 days of encapsulation. The results were normalized with those obtained with DS 0 group each day. Bar graphs symbolize the mean ± S.D (n=5). Statistical significance *$p < 0.05$ and ***$p < 0.001$ compared with DS 0 group.

Moreover, after 30 days of encapsulation we could clearly observe the trend of fibroblasts to form living cell aggregates in the presence of RGD (Fig. 3 I-L), while cells entrapped in no modified microcapsules remained with the same distribution observed at day 1. This finding come along with other previously reported results in the literature revealing that this cell line tends to form cell aggregates in the presence of adhesion ligands. Extensive research has been carried out regarding the formation of multicellular structures and the direct relation between the high proliferation activity of fibroblasts and the increase of the therapeutic factor expression ability [29,30].

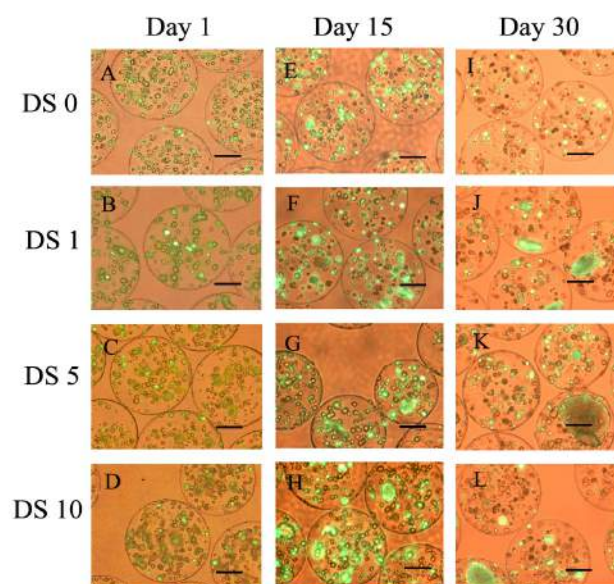


Figure 3. Fluorescence micrographs taken by day 1 (A-D), day 15 (E-H) and day 30 (I-L). Scale bars = 100 µm.

The results collected in this study show that maintaining the same number of encapsulated cells, the production of therapeutic factor may increase. However, it is important to notice that the enhancement of that secretion by the inclusion of this type of moiety may occur in an uncontrolled way, and in some cases this fact could result in appearance of side effects [31]. In this sense, the secretion obtained over a month by the DS 5 group may be excessive, and an exhaustive *in vivo* evaluation is necessary to estimate the dose required to achieve the best results.

In summary, these preliminary results suggest that RGD density is an important factor to take into account in the design of drug delivery biosystems. In the present work intermediate RGD density shows the greatest influence on the behavior of encapsulated fibroblasts. However, the role of RGD over cell behavior should be evaluated individually and carefully for each cell line, since its effects may be different depending on the cell type.

2. Microenvironment influence: *In vitro* and *in vivo* differences evaluated in EPO releasing C_2C_{12} myoblasts.

In order to prove the effect of RGD in other cell type which has also been widely used in some previous works as well, we carried out a similar experiment employing EPO secreting C_2C_{12} myoblasts in this case. In addition, in the present study we tried to compare the results collected *in vitro* with the effect of RGD-modified alginate on encapsulated cells in a complex natural microenvironment using an animal model.

Indeed, despite the demonstrated potency of this peptide sequence as bioactive molecule, recent investigations have shed controversial results concerning the effect of this adhesion moiety *in vivo*, opening an extended debate about its use [8,9,32,33]. This fact makes the so far used *in vitro* methods unreliable reporters of *in vivo* activity and, thereby, highlights the need for more *in vivo* studies. In this sense, researchers in the field are currently discussing the diverse factors that may influence in this lack of consistency between *in vitro* and *in vivo* results, including the background produced by the serum proteins adsorbed in the matrix [24,34] or the synergistic effect mediated by the different physicochemical cues coming from the surrounded microenvironment [35,36]. We used alginate-poly-l-lysine-alginate (APA) microcapsules as model for an *in vitro*-*in vivo* comparative study, since the semipermeable PLL membrane avoids/prevents the possible diffusion of high molecular weight serum proteins from the surrounding microenvironment [37].

The analyses of actin filaments *in vitro* suggested that immobilized cells were able to establish interaction sites with alginate in microcapsules containing RGD, whereas cells enclosed within alginate matrices without RGD remained with a rounded-shape (Fig. 4).

Even if these filopodia-like extensions, indicators of cell spreading, were more prominent as RGD density increased, the highest viability, proliferation and EPO secretion were obtained in the case of microcapsules elaborated with the lowest RGD density (DS 1 microcapsules) (Fig. 5). It is important to note that comparing with fibroblasts, myoblasts required a lower RGD densitiy to achieve the optimal functionality in those systems *in vitro*.

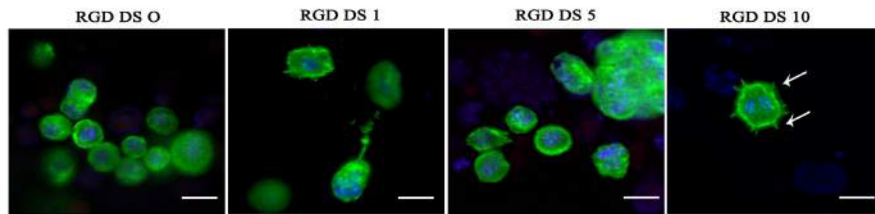


Figure 4. Cytoskeleton organization of myoblasts encapsulated in microcapsules elaborated with four different types of alginate *in vitro*. The cells inside APA microcapsules were stained with phalloidin Alexa Fluor 488 for F-actin (green) and Hoechst (blue) for nucleus. Scale bars = 20 μm.

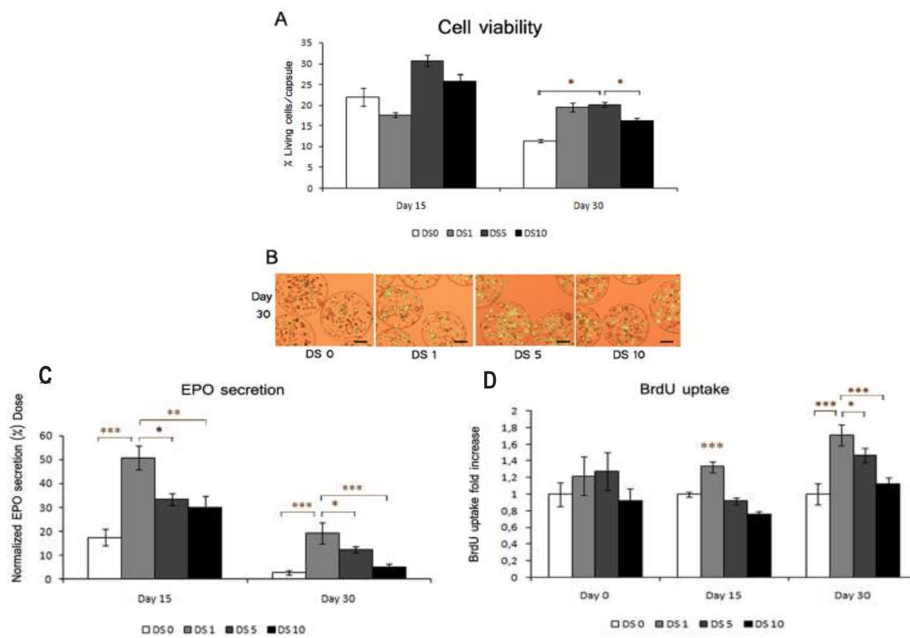


Figure 5. (A) *In vitro* percentage of living cells after 15 and 30 days of encapsulation. The number of living cells obtained for the day 0 was considered as 100% in each microcapsules group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). (B) *In vitro* Fluorescence micrographs taken by day 30. Scale bars = 100 μm. (C) *In vitro* EPO secretion after 15 and 30 days of encapsulation. Therapeutic factor secretion levels obtained for the day 0 were considered as 100% in each microcapsule group, and all values were expressed in function of this percentage. Bar graphs symbolize the mean ± S.D (n=3). (D) *In vitro* BrdU uptake after 0, 15 and 30 days of encapsulation. The results were normalized with those obtained with DS 0 group each day. Bar graphs symbolize the mean ± S.D (n=5). Statistical significance *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

We next moved on to *in vivo* assays in order to test the influence of a physiological microenvironment on cells enclosed within RGD-enriched matrices. When all types of microcapsules were retrieved from Balb/c mice at day 15 or 30, there was no evidence of inflammation process neither differences on the volume or adherences. We repeated the same experimental procedure carried out *in vitro* measuring the viability, EPO secretion and proliferation of encapsulated cells and the differences between both types of studies by day 30 were collected in the Figure 6.

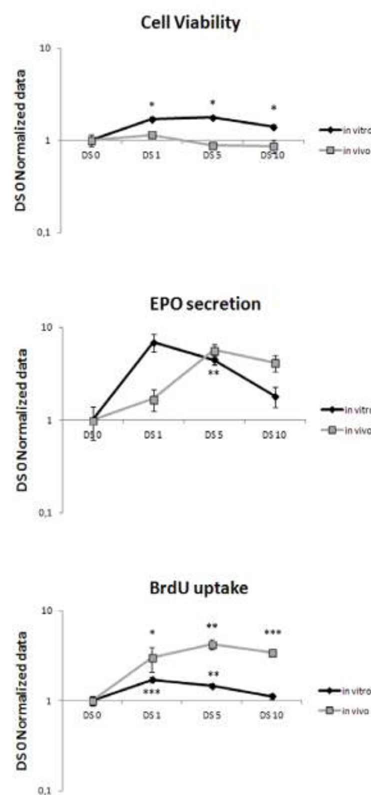


Figure 6. Direct comparison between *in vitro* and *in vivo* studies showing the synergistic effect of the RGD density and the microenvironment on cell viability, EPO secretion and BrdU uptake. The data obtained in either *in vitro* or *in vivo* studies were normalized against their respective DS 0 control group in order to compare the behavior of encapsulated cells in these two microenvironments in function of RGD density (DS 1, DS 5 and DS 10). Bar graphs symbolize the mean ± S.D (Standard deviation is within the size of the symbols in the graph). Statistical significance *p < 0.05, **p < 0.01 and ***p < 0.001; DS 0 vs other groups (DS1, DS5 and DS 10).

Higher RGD densities seem to be required *in vivo* to obtain the same effects observed *in vitro*. Graph curves for all assayed parameters revealed clearly that enclosed cells reflected almost the same behavior shown *in vitro* but displaced to higher densities of RGD. This also includes the inhibitory effect produced at the highest RGD density (DS 10). In the particular case of proliferation, it must be taken into account that the inclusion of RGD led to a higher proliferation activity in all types of microcapsules *in vivo*, compared to the lower values obtained *in vitro*. These differences may be attributable to the complexity provided by the *in vivo* body fluids to the microenvironment where microcapsules reside, which may also influence the final outcome of RGD on encapsulated cells. In fact, it is well known that several growth factors and hormones may alter the integrin expression of cells, and that their receptors cooperate with integrins in the regulation of adhesion-mediated signaling networks [35,36,38]. Therefore, special attention must be paid to the synergistic effect of the molecules coming from surrounding microenvironment, as this latter may vary according to the implantation site.

Some important parameters for the design of biomimetic biomaterials such as optimal RGD density and the influence of the surrounded microenvironment are presented in this study. Although further investigation are needed to define the molecular and cellular basis of these observations, these types of screening studies provide meaningful information in order to explore the complexity entailed by cell-ECM interaction. Likewise, future studies should be focused on studying the efficacy of RGD taking into account other parameters such as cell type or implantation site in the animal.

3. RGD influence on EPO releasing D1-MSCs: A step towards clinical application

In the last work of this doctoral thesis we evaluated the potential of our RGD-modified alginate microcapsules immobilizing D1 mesenchymal stem cells able to secrete EPO. Since the emergence of stem cells for cell-based therapies, special attention has been given to the use of this cell type also in the microencapsulation technology. This is due to the hipoimmunogenic properties of stem cells which can be of particular interest in the

design of 3D scaffolds to be implanted in nonautologous recipients. Despite the design of alginate-poly-l-lysine-alginate (APA) microcapsules protecting the implanted cells from the host immune response, the encapsulated nonautologous cells secrete cytokines and shed antigens. These factors may eventually evoke a host immune response giving rise to an inflammatory tissue surrounding the microcapsules that leads to suffocation and death of the encapsulated cells [39,40]. The use of MSCs that can downregulate or reduce this immune response at least locally has involved a great advance on the field, offering promising properties for cell microencapsulation and other cell-based therapies [41,42]. Besides the evaluation of D1-MSCs functionality immobilized in RGD-modified scaffolds, the aim of this work was to observe if these cells are able to maintain their stem cells properties when they are immobilized into RGD-modified alginate microcapsules.

After the phenotypic analysis and examination of differentiation potential of D1-MSCs, cells were immobilized into alginate scaffolds with different RGD densities. Cells immobilized into all types of microcapsules were able to maintain a high viability (75-80%) with slight differences between microcapsules elaborated with or without RGD. This could be explained by the fact that while some cell types need interactions for the attachment of the inner core, cell anchorage is not a strict requirement for survival of D1-MSCs [43]. Nevertheless, the results shown in the Figure 7C and 7D revealed that RGD added to the alginate matrices plays critical roles in some important cellular functions. According to the results collected in other works, the inclusion of RGD leads to an enhancement of the proliferation of encapsulated cells being the low and intermediate levels of RGD (DS 1 and DS 5) the most effective to obtain the highest proliferation activity and EPO secretion [44-46]. It should be noted that EPO secretion increases over time in all conditions, regardless of the RGD modification. The constant viability values obtained during all the experiment lead to the hypothesis that the enhancement of EPO released from D1-MSCs can be related with a higher functionality of enclosed cells.

The differentiation potential of encapsulated cells upon specific induction was confirmed by the presence of calcium deposits (osteogenic lineage) and lipid vacuoles (adipogenic lineage) in all types of microcapsules (Fig. 8B and 8C). The photographs of the Figures 8B and 8C belong to the DS 0 group. There were selected as example since the photographs

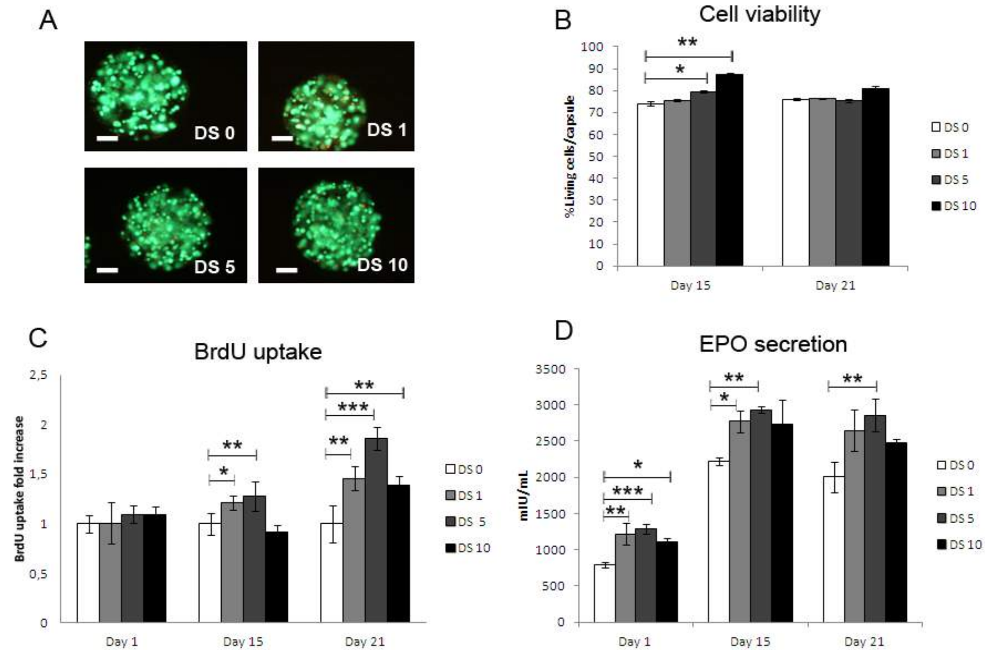


Figure 7. Fluorescence micrographs of cells encapsulated into different alginate matrices taken at day 21 (A). Percentage of living cells after 15 and 21 days of encapsulation (B). BrdU uptake (C) and EPO secretion (D) at day 1, 15 and 21 of the study. Bar graphs symbolize the mean ± S.D . Statistical significance *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with DS 0 group. Scale bars = 100 µm.

of the rest of the groups were similar (data not shown). However, after the absorbance measurement of the extracts obtained from de-encapsulated cells slight differences could be observed between evaluated groups (Fig. 8D). Our findings come along with other studies which reported that apart from its influence on cell functinality, RGD also promotes the differentiation of MSCs immobilized into 3D scaffolds [28,47].

Interestingly, RGD-modified alginate matrices did not provide a suitable environment for chondrogenic differentiation of D1-MSCs as observed in previous studies since cell aggregates are necessary to induce chondrogenic differentiation in MSCs in 3D cultures [42,48] and the high restriction forces of the solid environment within APA microcapsules does not facilitate that process.

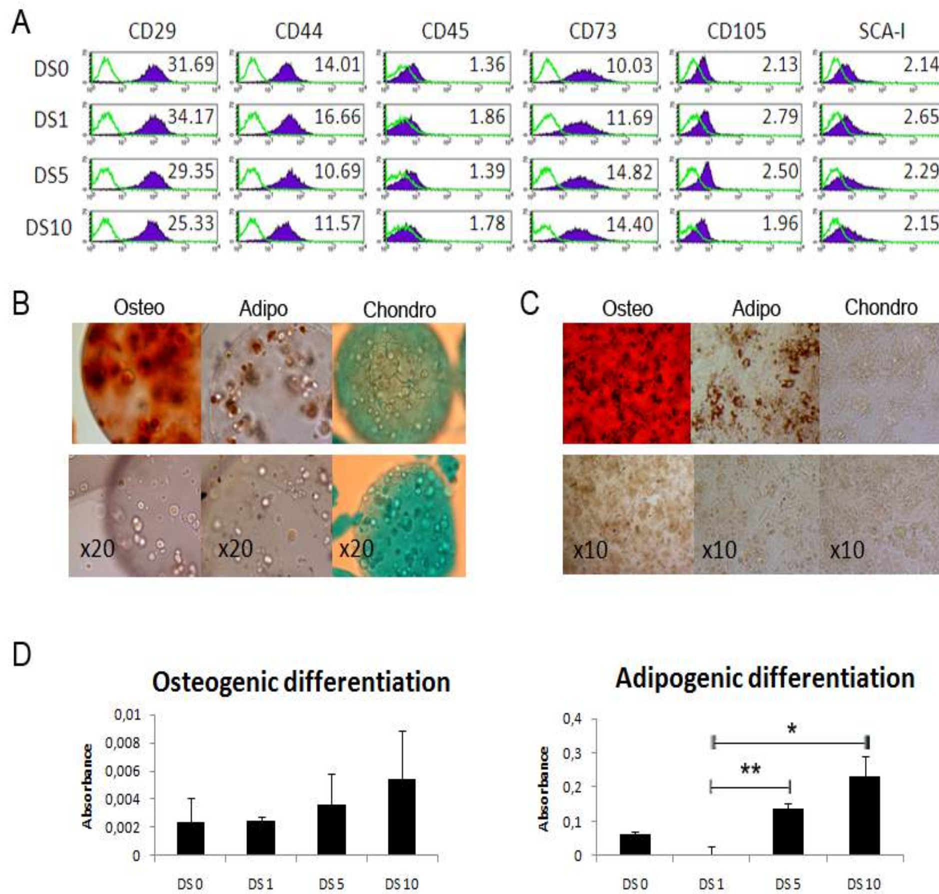


Figure 8. Phenotypic characterization of de-encapsulated D1-MSCs from all type of alginate matrices by FACS. Representative graphs of cell markers expression (filled histograms) and their isotype control (green lined histograms) are shown. Values in the graphs indicate the expression level represented as Mean Relative Fluorescence Intensity (MRFI), calculated by dividing the Mean Fluorescence Intensity (MFI) by its negative control (A). Diferentiation potential of microencapsulated D1-MSCs cultured 21 days in conditioned media. Osteogenic, adipogenic and chondrogenic differentiation with their respective negative controls within microcapsules (B) and after de-encapsulation (C). Quantification of osteogenic and adipogenic differentiation quantified by determining the absorbance of the extracts at 490 nm (D). Bar graphs symbolize the mean ± S.D. Statistical significance *$p < 0.05$ and **$p < 0.01$.

In a second set of experiments, we moved on to an *in vivo* assay to evaluate the behavior of D1-MSCs entrapped in different scaffolds in an allogenic recipient The elevated and similar hematocrit level obtained by all groups suggests that the fact of adding RGD into alginate does not have a significant effect on the EPO secretion (Fig. 9).

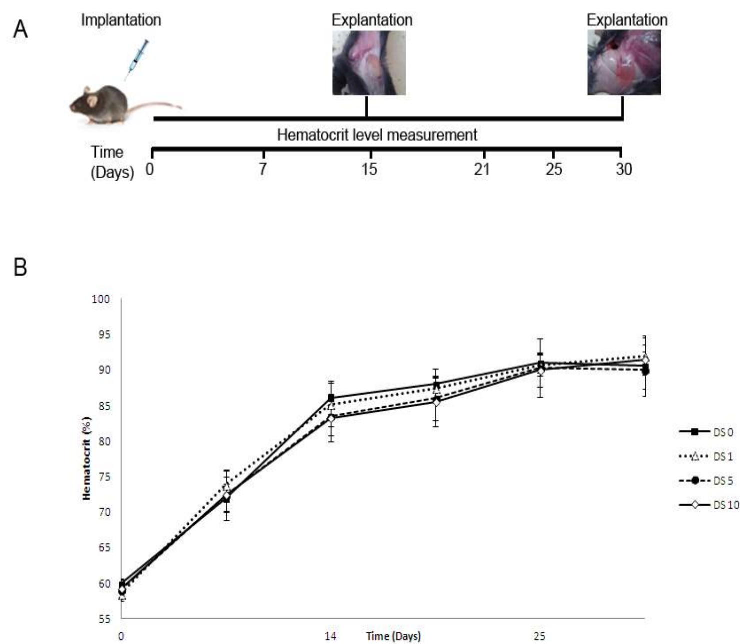


Figure 9. Schematic illustration of the *in vivo* study showing different time points for microcapsule explantation and hematocrit level mesurements (A). Hematocrit levels of C57BL/6J mice after subcutaneous implantation of EPO secreting D1-MSCs immobilized in different matrices (DS 0, DS 1, DS 5 and DS 10) (B).

To provide a more consistent data regarding that issue, EPO released by cells was analyzed after the explantation of microcapsules from the animals. No statistical differences between EPO secreted by D1-MSCs immobilized in different types of microcapsules were observed (Fig. 10B and 11B). However, it is important to note that although EPO levels were lower than those obtained in the *in vitro* assay, the implanted D1-MSCs also increased

EPO secretion over time in all conditions *in vivo*. The aggregates that these cells form into all types of microcapsules by day 30 (Fig. 11A) give us an idea of the possible reason to detect that enhancement of the therapeutic factor secretion during the 30 days period of the study [30]. Nevertheless, although EPO released from all microcapsules explanted at day 15 was very similar, at the end of the study some differences were detected between different groups, obtaining the highest EPO secretion levels in the microcapsules with low and intermediate densities of RGD (DS 1 and DS 5).

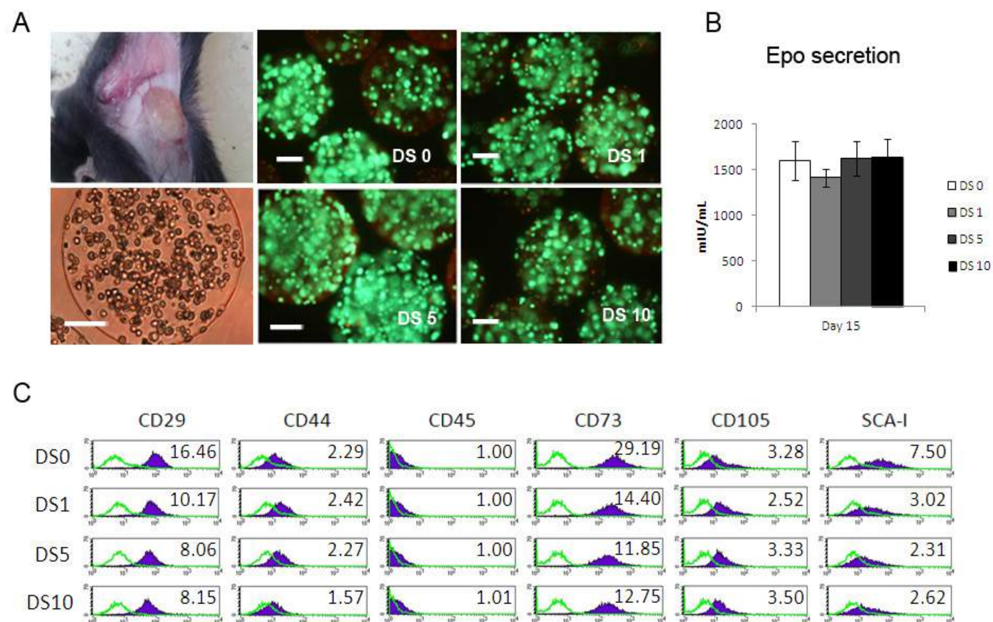


Figure 10. Microcapsules explanted at day 15: morphology of D1-MSCs and fluorescence micrographs of cells encapsulated into different alginate matrices taken at day 15 after implantation (A). EPO secreted of encapsulated cells after retrieval of mice at day 15 (B) Phenotypic characterization of D1-MSCs de-encapsulated of all type of alginate matrices at day 15 by FACS. Representative graphs of cell markers expression (filled histograms) and their isotype control (green lined histograms) are shown. Values in the graphs indicate the expression level represented as Mean Relative Fluorescence Intensity (MRFI), calculated by dividing the Mean Fluorescence Intensity (MFI) by its negative control (C).

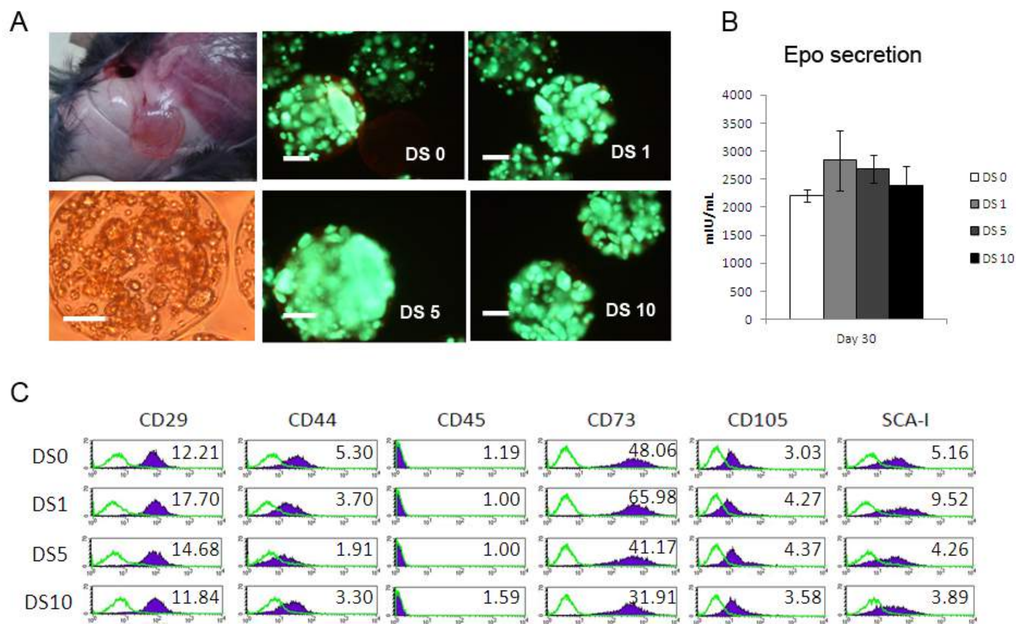


Figure 11. Microcapsules explanted at day 30: morphology of D1-MSCs and fluorescence micrographs of cells encapsulated into different alginate matrices taken at day 30 (A). EPO secreted of encapsulated cells after retrieval of mice at day 30 after implantation (B) Phenotypic characterization of D1-MSCs de-encapsulated of all type of alginate matrices at day 30 by FACS. Representative graphs of cell markers expression (filled histograms) and their isotype control (green lined histograms) are shown. Values in the graphs indicate the expression level represented as Mean Relative Fluorescence Intensity (MRFI), calculated by dividing the Mean Fluorescence Intensity (MFI) by its negative control (C).

The simultaneous analysis of a wide range of stem cell-related markers demonstrated that D1-MSCs immobilized into all microcapsules and cultured in basal media without differentiation induction were similar in terms of surface markers expression (Fig. 8A). According to the phenotypic analysis, it can be assumed that encapsulation of these cells into different matrices did not induce the differentiation towards adipo-, osteo- and chondrogenic lineages. Indeed, although the differentiation process may occur *in vivo* under certain circumstances [43], our *in vivo* experiments confirmed that MSCs remained

in an undifferentiated state maintaining their self-renewal potential (Fig. 10C and 11C).

The results collected in the present study indicate that the inclusion of RGD into alginate matrices do not affect the EPO secretion of encapsulated cells *in vivo* as much as in the *in vitro* study during the period of 30 days. The previous mentioned aggregates of D1-MSCs observed in the microcaspules implanted in mice revealed that proliferation increases even within microcapsules elaborated with no modified alginate. This suggests that the complexity provided by the *in vivo* body fluids to the microcapsules environment may have more influence that the fact of adding adhesion moieties into the matrices for this cell type. However, it should be highlighted that a longer *in vivo* follow-up period of the implanted microcapsules could allow observing more differences between different groups [10].

Altogether, these works demonstrate that RGD is an effective bioactive molecule which affects the behavior of immobilized cells. However, a judicious choice of ligand density is critical for the efficacy of this therapeutic strategy and tailoring the properties of the scaffold to every cell will result of paramount importance to obtain improved and more controllable results with increased biosafety.

References

1 Murua A, de Castro M, Orive G, Hernandez RM, Pedraz JL. *In vitro* characterization and *in vivo* functionality of erythropoietin-secreting cells immobilized in alginate-poly-L-lysine-alginate microcapsules. Biomacromolecules 8, 3302-3307 (2007).

2 Ngoc PK, Phuc PV, Nhung TH, Thuy DT, Nguyet NT. Improving the efficacy of type 1 diabetes therapy by transplantation of immunoisolated insulin-producing cells. Hum.Cell 24, 86-95 (2011).

3 Luca G, Fallarino F, Calvitti M, et al. Xenograft of microencapsulated sertoli cells reverses T1DM in NOD mice by inducing neogenesis of beta-cells. Transplantation 90, 1352-1357 (2010).

4 Klinge PM, Harmening K, Miller MC, et al. Encapsulated native and glucagon-like

peptide-1 transfected human mesenchymal stem cells in a transgenic mouse model of Alzheimer's disease. Neurosci.Lett. 497, 6-10 (2011).

5 Grandoso L, Ponce S, Manuel I, et al. Long-term survival of encapsulated GDNF secreting cells implanted within the striatum of parkinsonized rats. Int.J.Pharm. 343, 69-78 (2007).

6 Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog.Polym.Sci. 37, 106-126 (2012).

7 Augst AD, Kong HJ, Mooney DJ. Alginate hydrogels as biomaterials. Macromol.Biosci. 6, 623-633 (2006).

8 Collier JH, Segura T. Evolving the use of peptides as components of biomaterials. Biomaterials 32, 4198-4204 (2011).

9 Williams DF. The role of short synthetic adhesion peptides in regenerative medicine; the debate. Biomaterials 32, 4195-4197 (2011).

10 Orive G, De Castro M, Kong HJ, et al. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. J.Control.Release 135, 203-210 (2009).

11 Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 24, 4385-4415 (2003).

12 Hsiong SX, Huebsch N, Fischbach C, Kong HJ, Mooney DJ. Integrin-adhesion ligand bond formation of preosteoblasts and stem cells in three-dimensional RGD presenting matrices. Biomacromolecules 9, 1843-1851 (2008).

13 Wang X, Ye K, Li Z, Yan C, Ding J. Adhesion, proliferation, and differentiation of mesenchymal stem cells on RGD nanopatterns of varied nanospacings. Organogenesis 9, (2013).

14 Xiang M, Lin Y, He G, et al. Correlation between biological activity and binding energy in systems of integrin with cyclic RGD-containing binders: a QM/MM molecular dynamics study. J.Mol.Model. 18, 4917-4927 (2012).

15 Ruoslahti E. RGD and other recognition sequences for integrins. Annu.Rev.Cell Dev.

Biol. 12, 697-715 (1996).

16 Benoit YD, Groulx JF, Gagne D, Beaulieu JF. RGD-Dependent Epithelial Cell-Matrix Interactions in the Human Intestinal Crypt. J.Signal.Transduct 2012, 248759 (2012).

17 D'Souza SE, Ginsberg MH, Plow EF. Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. Trends Biochem.Sci. 16, 246-250 (1991).

18 Bidarra SJ, Barrias CC, Fonseca KB, Barbosa MA, Soares RA, Granja PL. Injectable in situ crosslinkable RGD-modified alginate matrix for endothelial cells delivery. Biomaterials 32, 7897-7904 (2011).

19 Connelly JT, Garcia AJ, Levenston ME. Inhibition of *in vitro* chondrogenesis in RGD-modified three-dimensional alginate gels. Biomaterials 28, 1071-1083 (2007).

20 Kim SY, Oh HK, Ha JM, et al. RGD-peptide presents anti-adhesive effect, but not direct pro-apoptotic effect on endothelial progenitor cells. Arch.Biochem.Biophys. 459, 40-49 (2007).

21 Lagunas A, Comelles J, Martinez E, et al. Cell adhesion and focal contact formation on linear RGD molecular gradients: study of non-linear concentration dependence effects. Nanomedicine 8, 432-439 (2012).

22 Re'em T, Tsur-Gang O, Cohen S. The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGFbeta1-induced chondrogenesis of human mesenchymal stem cells. Biomaterials 31, 6746-6755 (2010).

23 Wang X, Yan C, Ye K, He Y, Li Z, Ding J. Effect of RGD nanospacing on differentiation of stem cells. Biomaterials 34, 2865-2874 (2013).

24 Roach P, Farrar D, Perry CC. Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. J.Am.Chem.Soc. 128, 3939-3945 (2006).

25 Pierschbacher MD, Ruoslahti E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. J.Biol.Chem. 262, 17294-17298 (1987).

26 Houseman BT, Mrksich M. The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion. Biomaterials 22, 943-955 (2001).

27 Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. J.Biomed.Mater.Res. 60, 217-223 (2002).

28 Evangelista MB, Hsiong SX, Fernandes R, et al. Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. Biomaterials 28, 3644-3655 (2007).

29 Dai W, Saltzman WM. Fibroblast aggregation by suspension with conjugates of poly(ethylene glycol) and RGD. Biotechnol.Bioeng. 50, 349-356 (1996).

30 Jiang LY, Lv B, Luo Y. The effects of an RGD-PAMAM dendrimer conjugate in 3D spheroid culture on cell proliferation, expression and aggregation. Biomaterials 34, 2665-2673 (2013).

31 Yasuhara T, Shingo T, Muraoka K, et al. Neurorescue effects of VEGF on a rat model of Parkinson's disease. Brain Res. 1053, 10-18 (2005).

32 Bellis SL. Advantages of RGD peptides for directing cell association with biomaterials. Biomaterials 32, 4205-4210 (2011).

33 Barker TH. The role of ECM proteins and protein fragments in guiding cell behavior in regenerative medicine. Biomaterials 32, 4211-4214 (2011).

34 Allen LT, Tosetto M, Miller IS, et al. Surface-induced changes in protein adsorption and implications for cellular phenotypic responses to surface interaction. Biomaterials 27, 3096-3108 (2006).

35 Sinha RK, Tuan RS. Regulation of human osteoblast integrin expression by orthopedic implant materials. Bone 18, 451-457 (1996).

36 Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. J.Biol.Chem. 264, 380-388 (1989).

37 Leung A, Nielsen L, Trau M, Timmins N. Tissue transplantation by stealth-coherent alginate microcapsules for immunoisolation. Biochem Eng J 48, 337-47 (2010).

38 Plopper GE, McNamee HP, Dike LE, Bojanowski K, Ingber DE. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol. Biol.Cell 6, 1349-1365 (1995).

39 Orive G, Tam SK, Pedraz JL, Halle JP. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. Biomaterials 27, 3691-3700 (2006).

40 de Groot M, Schuurs TA, van Schilfgaarde R. Causes of limited survival of microencapsulated pancreatic islet grafts. J.Surg.Res. 121, 141-150 (2004).

41 Liu H, Lu K, Macary PA, et al. Soluble molecules are key in maintaining the immunomodulatory activity of murine mesenchymal stromal cells. J.Cell.Sci. 125, 200-208 (2012).

42 Goren A, Dahan N, Goren E, Baruch L, Machluf M. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. FASEB J. 24, 22-31 (2010).

43 Juffroy O, Noel D, Delanoye A, Viltart O, Wolowczuk I, Verwaerde C. Subcutaneous graft of D1 mouse mesenchymal stem cells leads to the formation of a bone-like structure. Differentiation 78, 223-231 (2009).

44 Neff JA, Tresco PA, Caldwell KD. Surface modification for controlled studies of cell-ligand interactions. Biomaterials 20, 2377-2393 (1999).

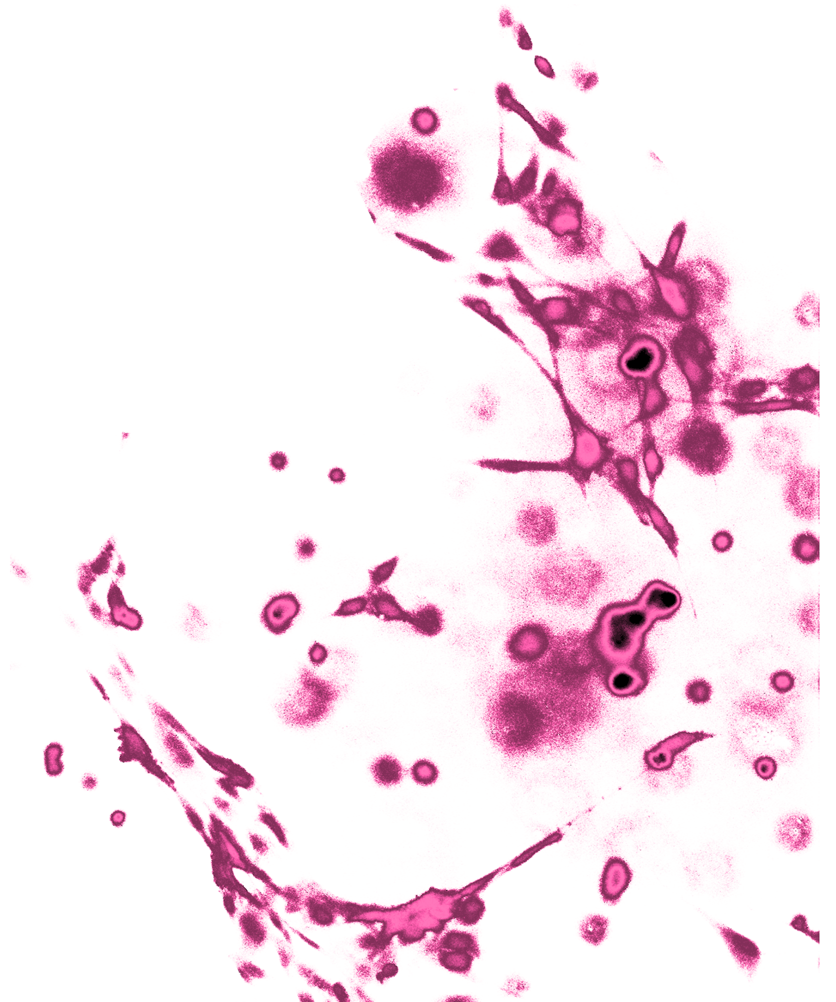
45 Garate A, Santos E, Pedraz JL, Hernandez RM, Orive G. Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems. J.Drug Target. 1-7 (2015).

46 Santos E, Garate A, Pedraz JL, Orive G, Hernandez RM. The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells: *In vitro* and *in vivo* direct comparative study. J.Biomed.Mater.Res.A. (2013).

47 Grellier M, Granja PL, Fricain JC, et al. The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on mineralization in a bone defect. Biomaterials 30, 3271-3278 (2009).

48 Sayyar B, Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Fibronectin-Alginate microcapsules improve cell viability and protein secretion of encapsulated Factor IX-engineered human mesenchymal stromal cells. Artif.Cells Nanomed Biotechnol. (2014).

Conclusions



According to the results obtained in the present experimental work, the main conclusions include:

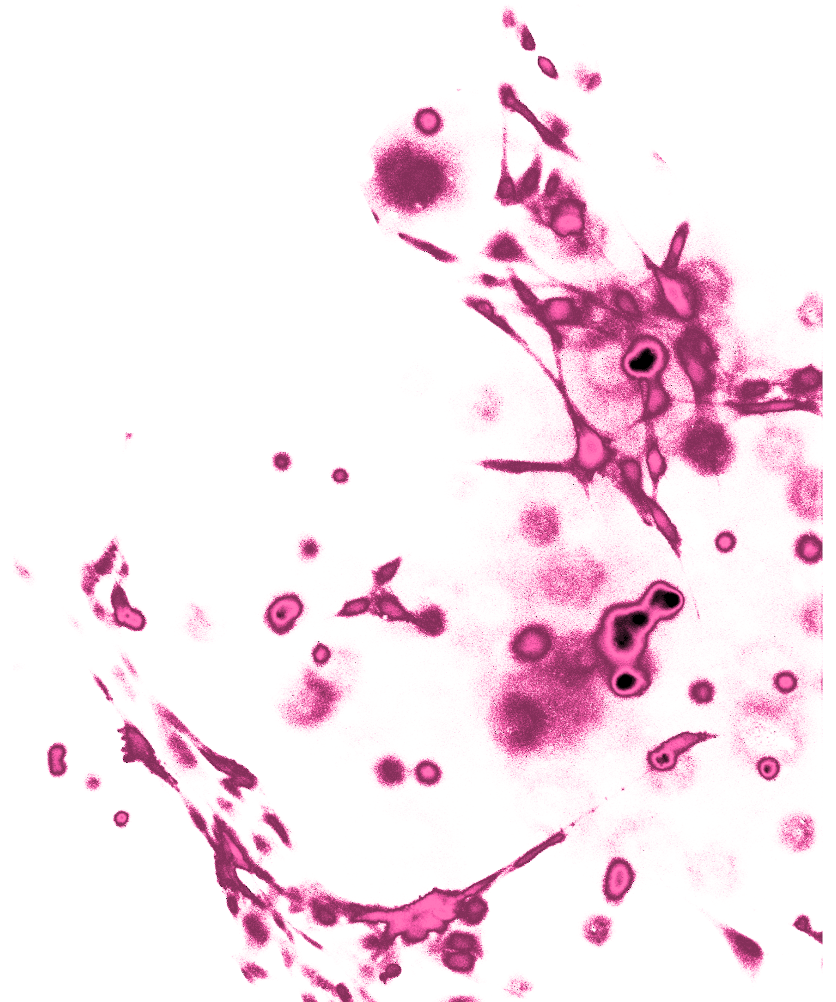
- The modification of alginate microcapsules with different RGD densities affects the behavior of immobilized cells in terms of viability, proliferation and/or therapeutic factor secretion. In general, low or intermediate RGD densities seem to have more influence on the enhancement of cell activity than the highest RGD density.

- Cells enclosed within microcapsules elaborated with RGD show higher viability and proliferation *in vitro*. Regarding the therapeutic factor secretion, a higher release is observed in microcapsules with RGD, although in the case of fibroblasts the differences were not statistically significant.

- The *in vivo* study carried out with myoblasts reflects a similar enhancement of EPO secretion and proliferation of immobilized cells as observed *in vitro*. However, the aggregates that D1-MSCs formed *in vivo* in all types of microcapsules probably have hindered the observation of RGD effects in this cell type.

- The study carried out with MSCs demonstrates that subcutaneous implantation of microcapsules does not induce differentiation of D1-MSCs towards any lineage remaining at an undifferentiated state *in vivo* during this study.

Bibliography



Acarregui A, Murua A, Pedraz JL, Orive G, Hernandez RM. A perspective on bioactive cell microencapsulation. BioDrugs 26, 283-301 (2012).

Al Kindi AH, Asenjo JF, Ge Y, et al. Microencapsulation to reduce mechanical loss of microspheres: implications in myocardial cell therapy. Eur.J.Cardiothorac.Surg. 39, 241-247 (2011).

Alsberg E, Anderson KW, Albeiruti A, Rowley JA, Mooney DJ. Engineering growing tissues. Proc. Natl.Acad.Sci.U.S.A. 99, 12025-12030 (2002).

Allen LT, Tosetto M, Miller IS, et al. Surface-induced changes in protein adsorption and implications for cellular phenotypic responses to surface interaction. Biomaterials 27, 3096-3108 (2006).

Amado LC, Saliaris AP, Schuleri KH, et al. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc.Natl.Acad.Sci.U.S.A. 102, 11474-11479 (2005).

Amsden B, Turner N. Diffusion characteristics of calcium alginate gels. Biotechnol.Bioeng. 65, 605-610 (1999).

Aota S, Nagai T, Yamada KM. Characterization of regions of fibronectin besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using site-directed mutagenesis. J.Biol.Chem. 266, 15938-15943 (1991).

Arnaout MA, Mahalingam B, Xiong JP. Integrin structure, allostery, and bidirectional signaling. Annu.Rev.Cell Dev.Biol. 21, 381-410 (2005).

Ashton RS, Banerjee A, Punyani S, Schaffer DV, Kane RS. Scaffolds based on degradable alginate hydrogels and poly(lactide-co-glycolide) microspheres for stem cell culture. Biomaterials 28, 5518-5525 (2007).

Attia N, Santos E, Abdelmouty H, et al. Behaviour and ultrastructure of human bone marrow-derived mesenchymal stem cells immobilised in alginate-poly-l-lysine-alginate microcapsules. J.Microencapsul. 31, 579-589 (2014).

Augello A, Kurth TB, De Bari C. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. Eur.Cell.Mater. 20, 121-133 (2010).

Augst AD, Kong HJ, Mooney DJ. Alginate hydrogels as biomaterials. Macromol.Biosci. 6, 623-633 (2006).

Bae SE, Choi DH, Han DK, Park K. Effect of temporally controlled release of dexamethasone on in vivo chondrogenic differentiation of mesenchymal stromal cells. J.Control.Release 143, 23-30 (2010).

Bai HY, Chen GA, Mao GH, Song TR, Wang YX. Three step derivation of cartilage like tissue from human embryonic stem cells by 2D-3D sequential culture in vitro and further implantation in vivo on alginate/PLGA scaffolds. J.Biomed.Mater.Res.A. 94, 539-546 (2010).

Banerjee A, Arha M, Choudhary S, et al. The influence of hydrogel modulus on the proliferation and differentiation of encapsulated neural stem cells. Biomaterials 30, 4695-4699 (2009).

Barbero A, Grogan S, Schafer D, Heberer M, Mainil-Varlet P, Martin I. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. Osteoarthritis Cartilage 12, 476-484 (2004).

Barczyk M, Carracedo S, Gullberg D. Integrins. Cell Tissue Res. 339, 269-280 (2010).

Barker TH. The role of ECM proteins and protein fragments in guiding cell behavior in regenerative medicine. Biomaterials 32, 4211-4214 (2011).

Bellis SL. Advantages of RGD peptides for directing cell association with biomaterials. Biomaterials 32, 4205-4210 (2011).

Beltrami AP, Cesselli D, Beltrami CA. Stem cell senescence and regenerative paradigms. Clin. Pharmacol.Ther. 91, 21-29 (2012).

Benoit DS, Schwartz MP, Durney AR, Anseth KS. Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells. Nat.Mater. 7, 816-823 (2008).

Benoit YD, Groulx JF, Gagne D, Beaulieu JF. RGD-Dependent Epithelial Cell-Matrix Interactions in the Human Intestinal Crypt. J.Signal.Transduct 2012, 248759 (2012).

Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood 105, 2214-2219 (2005).

Bhat A, Hoch AI, Decaris ML, Leach JK. Alginate hydrogels containing cell-interactive beads for bone formation. FASEB J. 27, 4844-4852 (2013).

Bian L, Zhai DY, Tous E, Rai R, Mauck RL, Burdick JA. Enhanced MSC chondrogenesis following delivery of TGF-beta3 from alginate microspheres within hyaluronic acid hydrogels in vitro and in vivo. Biomaterials 32, 6425-6434 (2011).

Bidarra SJ, Barrias CC, Barbosa MA, Soares R, Granja PL. Immobilization of human mesenchymal stem cells within RGD-grafted alginate microspheres and assessment of their angiogenic potential. Biomacromolecules 11, 1956-1964 (2010).

Bidarra SJ, Barrias CC, Fonseca KB, Barbosa MA, Soares RA, Granja PL. Injectable in situ crosslinkable

RGD-modified alginate matrix for endothelial cells delivery. Biomaterials 32, 7897-7904 (2011).

Boomsma RA, Geenen DL. Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. PLoS One 7, e35685 (2012).

Boontheekul T, Kong HJ, Mooney DJ. Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. Biomaterials 26, 2455-2465 (2005).

Borlongan CV, Skinner SJ, Geaney M, Vasconcellos AV, Elliott RB, Emerich DF. Neuroprotection by encapsulated choroid plexus in a rodent model of Huntington's disease. Neuroreport 15, 2521-2525 (2004).

Bouhadir KH, Lee KY, Alsberg E, Damm KL, Anderson KW, Mooney DJ. Degradation of partially oxidized alginate and its potential application for tissue engineering. Biotechnol.Prog. 17, 945-950 (2001).

Bozza A, Coates EE, Incitti T, et al. Neural differentiation of pluripotent cells in 3D alginate-based cultures. Biomaterials 35, 4636-4645 (2014).

Buckley CT, Meyer EG, Kelly DJ. The influence of construct scale on the composition and functional properties of cartilaginous tissues engineered using bone marrow-derived mesenchymal stem cells. Tissue Eng.Part A. 18, 382-396 (2012).

Burridge PW, Keller G, Gold JD, Wu JC. Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. Cell.Stem Cell. 10, 16-28 (2012).

Buxboim A, Discher DE. Stem cells feel the difference. Nat.Methods 7, 695-697 (2010).

Cai J, Huang Y, Chen X, Xie H, Huang Y, Deng L. Regulation of sonic hedgehog on vascular endothelial growth factor, basic fibroblast growth factor expression and secretion in bone marrow mesenchymal stem cells. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 26, 112-116 (2012).

Calafiore R, Basta G, Luca G, et al. Standard technical procedures for microencapsulation of human islets for graft into nonimmunosuppressed patients with type 1 diabetes mellitus. Transplant.Proc. 38, 1156-1157 (2006).

Calafiore R, Basta G. Clinical application of microencapsulated islets: Actual prospectives on progress and challenges. Adv.Drug Deliv.Rev. (2013).

Candiello J, Singh SS, Task K, Kumta PN, Banerjee I. Early differentiation patterning of mouse embryonic stem cells in response to variations in alginate substrate stiffness. J.Biol.Eng. 7, 9-1611-7-9 (2013).

Casado JG, Tarazona R, Sanchez-Margallo FM. NK and MSCs crosstalk: the sense of immunomodulation and their sensitivity. Stem Cell.Rev. 9, 184-189 (2013).

Caterson EJ, Li WJ, Nesti LJ, Albert T, Danielson K, Tuan RS. Polymer/alginate amalgam for cartilage-tissue engineering. Ann.N.Y.Acad.Sci. 961, 134-138 (2002).

Chayosumrit M, Tuch B, Sidhu K. Alginate microcapsule for propagation and directed differentiation of hESCs to definitive endoderm. Biomaterials 31, 505-514 (2010).

Chen PM, Yen ML, Liu KJ, Sytwu HK, Yen BL. Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells. J.Biomed.Sci. 18, 49-0127-18-49 (2011).

Chen W, Zhou H, Tang M, Weir MD, Bao C, Xu HH. Gas-Foaming Calcium Phosphate Cement Scaffold Encapsulating Human Umbilical Cord Stem Cells. Tissue Eng.Part A. (2011).

Chiu RC. MSC immune tolerance in cellular cardiomyoplasty. Semin.Thorac.Cardiovasc.Surg. 20, 115-118 (2008).

Choi DH, Park CH, Kim IH, Chun HJ, Park K, Han DK. Fabrication of core-shell microcapsules using PLGA and alginate for dual growth factor delivery system. J.Control.Release 147, 193-201 (2010).

Christman KL, Vardanian AJ, Fang Q, Sievers RE, Fok HH, Lee RJ. Injectable fibrin scaffold improves cell transplant survival, reduces infarct expansion, and induces neovasculature formation in ischemic myocardium. J.Am.Coll.Cardiol. 44, 654-660 (2004).

Collier JH, Segura T. Evolving the use of peptides as components of biomaterials. Biomaterials 32, 4198-4204 (2011).

Collins VP, James CD. Gene and chromosomal alterations associated with the development of human gliomas. FASEB J. 7, 926-930 (1993).

Comisar WA, Hsiong SX, Kong HJ, Mooney DJ, Linderman JJ. Multi-scale modeling to predict ligand presentation within RGD nanopatterned hydrogels. Biomaterials 27, 2322-2329 (2006).

Comisar WA, Kazmers NH, Mooney DJ, Linderman JJ. Engineering RGD nanopatterned hydrogels to control preosteoblast behavior: a combined computational and experimental approach. Biomaterials 28, 4409-4417 (2007).

Connelly JT, Garcia AJ, Levenston ME. Inhibition of in vitro chondrogenesis in RGD-modified three-dimensional alginate gels. Biomaterials 28, 1071-1083 (2007).

Cortes-Morichetti M, Frati G, Schussler O, et al. Association between a cell-seeded collagen matrix and cellular cardiomyoplasty for myocardial support and regeneration. Tissue Eng. 13, 2681-2687

(2007).

Cunha C, Panseri S, Villa O, Silva D, Gelain F. 3D culture of adult mouse neural stem cells within functionalized self-assembling peptide scaffolds. Int.J.Nanomedicine 6, 943-955 (2011).

Dai W, Saltzman WM. Fibroblast aggregation by suspension with conjugates of poly(ethylene glycol) and RGD. Biotechnol.Bioeng. 50, 349-356 (1996).

Dalton PD, Mey J. Neural interactions with materials. Front.Biosci. 14, 769-795 (2009).

Das H, George JC, Joseph M, et al. Stem cell therapy with overexpressed VEGF and PDGF genes improves cardiac function in a rat infarct model. PLoS One 4, e7325 (2009).

Dashtdar H, Rothan HA, Tay T, et al. A preliminary study comparing the use of allogenic chondrogenic pre-differentiated and undifferentiated mesenchymal stem cells for the repair of full thickness articular cartilage defects in rabbits. J.Orthop.Res. 29, 1336-1342 (2011).

de Groot M, Schuurs TA, van Schilfgaarde R. Causes of limited survival of microencapsulated pancreatic islet grafts. J.Surg.Res. 121, 141-150 (2004).

de Haan BJ, Rossi A, Faas MM, et al. Structural surface changes and inflammatory responses against alginate-based microcapsules after exposure to human peritoneal fluid. J.Biomed.Mater.Res.A. 98, 394-403 (2011).

de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials 27, 5603-5617 (2006).

Deng Y, Zhou H, Yan C, et al. In vitro osteogenic induction of bone marrow stromal cells with encapsulated gene-modified bone marrow stromal cells and in vivo implantation for orbital bone repair. Tissue Eng.Part A. 20, 2019-2029 (2014).

Diduch DR, Jordan LC, Mierisch CM, Balian G. Marrow stromal cells embedded in alginate for repair of osteochondral defects. Arthroscopy 16, 571-577 (2000).

Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Sustained expression of coagulation factor IX by modified cord blood-derived mesenchymal stromal cells. J.Gene Med. 16, 131-142 (2014).

Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315-317 (2006).

Drury JL, Dennis RG, Mooney DJ. The tensile properties of alginate hydrogels. Biomaterials 25,

3187-3199 (2004).

D'Souza SE, Ginsberg MH, Plow EF. Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. Trends Biochem.Sci. 16, 246-250 (1991).

Dvir-Ginzberg M, Elkayam T, Cohen S. Induced differentiation and maturation of newborn liver cells into functional hepatic tissue in macroporous alginate scaffolds. FASEB J. 22, 1440-1449 (2008).

Eid K, Chen E, Griffith L, Glowacki J. Effect of RGD coating on osteocompatibility of PLGA-polymer disks in a rat tibial wound. J.Biomed.Mater.Res. 57, 224-231 (2001).

Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell 126, 677-689 (2006).

Evangelista MB, Hsiong SX, Fernandes R, et al. Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. Biomaterials 28, 3644-3655 (2007).

Facca S, Cortez C, Mendoza-Palomares C, et al. Active multilayered capsules for in vivo bone formation. Proc.Natl.Acad.Sci.U.S.A. 107, 3406-3411 (2010).

Ferris DM, Moodie GD, Dimond PM, Gioranni CW, Ehrlich MG, Valentini RF. RGD-coated titanium implants stimulate increased bone formation in vivo. Biomaterials 20, 2323-2331 (1999).

Fu Y, Azene N, Ehtiati T, et al. Fused X-ray and MR imaging guidance of intrapericardial delivery of microencapsulated human mesenchymal stem cells in immunocompetent swine. Radiology 272, 427-437 (2014).

Gabr MM, Sobh MM, Zakaria MM, Refaie AF, Ghoneim MA. Transplantation of insulin-producing clusters derived from adult bone marrow stem cells to treat diabetes in rats. Exp.Clin.Transplant. 6, 236-243 (2008).

Garate A, Santos E, Pedraz JL, Hernandez RM, Orive G. Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems. J.Drug Target. 1-7 (2015).

Gasperini L, Mano JF, Reis RL. Natural polymers for the microencapsulation of cells. J.R.Soc.Interface 11, 20140817 (2014).

Gehmert S, Gehmert S, Hidayat M, et al. Angiogenesis: the role of PDGF-BB on adiopse-tissue derived stem cells (ASCs). Clin.Hemorheol.Microcirc. 48, 5-13 (2011).

Glicklis R, Shapiro L, Agbaria R, Merchuk JC, Cohen S. Hepatocyte behavior within three-dimensional porous alginate scaffolds. Biotechnol.Bioeng. 67, 344-353 (2000).

Godfrey KJ, Mathew B, Bulman JC, Shah O, Clement S, Gallicano GI. Stem cell-based treatments for Type 1 diabetes mellitus: bone marrow, embryonic, hepatic, pancreatic and induced pluripotent stem cells. Diabet.Med. (2011).

Goren A, Dahan N, Goren E, Baruch L, Machluf M. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. FASEB J. 24, 22-31 (2010).

Grandoso L, Ponce S, Manuel I, et al. Long-term survival of encapsulated GDNF secreting cells implanted within the striatum of parkinsonized rats. Int.J.Pharm. 343, 69-78 (2007).

Grellier M, Granja PL, Fricain JC, et al. The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on mineralization in a bone defect. Biomaterials 30, 3271-3278 (2009).

Grigore A, Sarker B, Fabry B, Boccaccini AR, Detsch R. Behavior of encapsulated MG-63 cells in RGD and gelatine-modified alginate hydrogels. Tissue Eng.Part A. 20, 2140-2150 (2014).

Gugerli R, Cantana E, Heinzen C, von Stockar U, Marison IW. Quantitative study of the production and properties of alginate/poly-L-lysine microcapsules. J.Microencapsul. 19, 571-590 (2002).

Gurruchaga H, Ciriza J, Saenz Del Burgo L, et al. Cryopreservation of microencapsulated murine mesenchymal stem cells genetically engineered to secrete erythropoietin. Int.J.Pharm. 485, 15-24 (2015).

Gurruchaga H, Saenz Del Burgo L, Ciriza J, Orive G, Hernandez RM, Pedraz JL. Advances in cell encapsulation technology and its application in drug delivery. Expert Opin.Drug Deliv. 1-17 (2015).

Hamazaki T, Iiboshi Y, Oka M, et al. Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS Lett. 497, 15-19 (2001).

Heile A, Brinker T. Clinical translation of stem cell therapy in traumatic brain injury: the potential of encapsulated mesenchymal cell biodelivery of glucagon-like peptide-1. Dialogues Clin.Neurosci. 13, 279-286 (2011).

Heile AM, Wallrapp C, Klinge PM, et al. Cerebral transplantation of encapsulated mesenchymal stem cells improves cellular pathology after experimental traumatic brain injury. Neurosci.Lett. 463, 176-181 (2009).

Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. J.Biol.Chem. 264, 380-388 (1989).

Hernandez RM, Orive G, Murua A, Pedraz JL. Microcapsules and microcarriers for in situ cell delivery.

Adv.Drug Deliv.Rev. 62, 711-730 (2010).

Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 24, 4385-4415 (2003).

Hipp J, Atala A. Tissue engineering, stem cells, cloning, and parthenogenesis: new paradigms for therapy. J.Exp.Clin.Assist.Reprod. 1, 3 (2004).

Horner PJ, Gage FH. Regenerating the damaged central nervous system. Nature 407, 963-970 (2000).

Hortelano G, Al-Hendy A, Ofosu FA, Chang PL. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. Blood 87, 5095-5103 (1996).

Houseman BT, Mrksich M. The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion. Biomaterials 22, 943-955 (2001).

Hsiao ST, Asgari A, Lokmic Z, et al. Comparative Analysis of Paracrine Factor Expression in Human Adult Mesenchymal Stem Cells Derived from Bone Marrow, Adipose, and Dermal Tissue. Stem Cells Dev. (2012).

Hsiong SX, Huebsch N, Fischbach C, Kong HJ, Mooney DJ. Integrin-adhesion ligand bond formation of preosteoblasts and stem cells in three-dimensional RGD presenting matrices. Biomacromolecules 9, 1843-1851 (2008).

Huang S, Jia S, Liu G, Fang D, Zhang D. Osteogenic differentiation of muscle satellite cells induced by platelet-rich plasma encapsulated in three-dimensional alginate scaffold. Oral Surg.Oral Med. Oral Pathol.Oral Radiol.Endod. (2012).

Huang X, Zhang X, Wang X, Wang C, Tang B. Microenvironment of alginate-based microcapsules for cell culture and tissue engineering. J.Biosci.Bioeng. 114, 1-8 (2012).

Huang YC, Liu TJ. Mobilization of mesenchymal stem cells by stromal cell-derived factor-1 released from chitosan/tripolyphosphate/fucoidan nanoparticles. Acta Biomater. 8, 1048-1056 (2012).

Hui TY, Cheung KM, Cheung WL, Chan D, Chan BP. In vitro chondrogenic differentiation of human mesenchymal stem cells in collagen microspheres: influence of cell seeding density and collagen concentration. Biomaterials 29, 3201-3212 (2008).

Hwang YS, Cho J, Tay F, et al. The use of murine embryonic stem cells, alginate encapsulation, and rotary microgravity bioreactor in bone tissue engineering. Biomaterials 30, 499-507 (2009).

Jing D, Parikh A, Tzanakakis ES. Cardiac cell generation from encapsulated embryonic stem cells in static and scalable culture systems. Cell Transplant. 19, 1397-1412 (2010).

Jiang LY, Lv B, Luo Y. The effects of an RGD-PAMAM dendrimer conjugate in 3D spheroid culture on cell proliferation, expression and aggregation. Biomaterials 34, 2665-2673 (2013).

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp.Cell Res. 238, 265-272 (1998).

Juffroy O, Noel D, Delanoye A, Viltart O, Wolowczuk I, Verwaerde C. Subcutaneous graft of D1 mouse mesenchymal stem cells leads to the formation of a bone-like structure. Differentiation 78, 223-231 (2009).

Karoubi G, Ormiston ML, Stewart DJ, Courtman DW. Single-cell hydrogel encapsulation for enhanced survival of human marrow stromal cells. Biomaterials 30, 5445-5455 (2009).

Karsenty G. The complexities of skeletal biology. Nature 423, 316-318 (2003).

Kauer TM, Figueiredo JL, Hingtgen S, Shah K. Encapsulated therapeutic stem cells implanted in the tumor resection cavity induce cell death in gliomas. Nat.Neurosci. 15, 197-204 (2011).

Kavalkovich KW, Boynton RE, Murphy JM, Barry F. Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system. In Vitro Cell.Dev.Biol.Anim. 38, 457-466 (2002).

Kerby A, Jones ES, Jones PM, King AJ. Co-transplantation of islets with mesenchymal stem cells in microcapsules demonstrates graft outcome can be improved in an isolated-graft model of islet transplantation in mice. Cytotherapy 15, 192-200 (2013).

Khalil M, Shariat-Panahi A, Tootle R, et al. Human hepatocyte cell lines proliferating as cohesive spheroid colonies in alginate markedly upregulate both synthetic and detoxificatory liver function. J.Hepatol. 34, 68-77 (2001).

Kim SY, Oh HK, Ha JM, et al. RGD-peptide presents anti-adhesive effect, but not direct pro-apoptotic effect on endothelial progenitor cells. Arch.Biochem.Biophys. 459, 40-49 (2007).

King A, Sandler S, Andersson A. The effect of host factors and capsule composition on the cellular overgrowth on implanted alginate capsules. J.Biomed.Mater.Res. 57, 374-383 (2001).

Kisseleva T, Brenner DA. The phenotypic fate and functional role for bone marrow-derived stem cells in liver fibrosis. J.Hepatol. (2011).

Kleinschmidt K, Klinge PM, Stopa E, et al. Alginate encapsulated human mesenchymal stem cells

suppress syngeneic glioma growth in the immunocompetent rat. J.Microencapsul. 28, 621-627 (2011).

Klinge PM, Harmening K, Miller MC, et al. Encapsulated native and glucagon-like peptide-1 transfected human mesenchymal stem cells in a transgenic mouse model of Alzheimer's disease. Neurosci.Lett. 497, 6-10 (2011).

Kofidis T, Lebl DR, Martinez EC, Hoyt G, Tanaka M, Robbins RC. Novel injectable bioartificial tissue facilitates targeted, less invasive, large-scale tissue restoration on the beating heart after myocardial injury. Circulation 112, I173-7 (2005).

Kolambkar YM, Dupont KM, Boerckel JD, et al. An alginate-based hybrid system for growth factor delivery in the functional repair of large bone defects. Biomaterials 32, 65-74 (2011).

Kong HJ, Smith MK, Mooney DJ. Designing alginate hydrogels to maintain viability of immobilized cells. Biomaterials 24, 4023-4029 (2003).

Koo LY, Irvine DJ, Mayes AM, Lauffenburger DA, Griffith LG. Co-regulation of cell adhesion by nanoscale RGD organization and mechanical stimulus. J.Cell.Sci. 115, 1423-1433 (2002).

Kraehenbuehl TP, Ferreira LS, Hayward AM, et al. Human embryonic stem cell-derived microvascular grafts for cardiac tissue preservation after myocardial infarction. Biomaterials 32, 1102-1109 (2011).

Krishnamurthy NV, Gimi B. Encapsulated cell grafts to treat cellular deficiencies and dysfunction. Crit.Rev.Biomed.Eng. 39, 473-491 (2011).

Kronenberg HM. Developmental regulation of the growth plate. Nature 423, 332-336 (2003).

Lagunas A, Comelles J, Martinez E, et al. Cell adhesion and focal contact formation on linear RGD molecular gradients: study of non-linear concentration dependence effects. Nanomedicine 8, 432-439 (2012).

Lee KY, Peters MC, Anderson KW, Mooney DJ. Controlled growth factor release from synthetic extracellular matrices. Nature 408, 998-1000 (2000).

Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog.Polym.Sci. 37, 106-126 (2012).

Leipzig ND, Shoichet MS. The effect of substrate stiffness on adult neural stem cell behavior. Biomaterials 30, 6867-6878 (2009).

Leung A, Nielsen L, Trau M, Timmins N. Tissue transplantation by stealth-coherent alginate microcapsules for immunoisolation. Biochem Eng J 48, 337-47 (2010).

Li C, Zhao S, Zhao Y, Qian Y, Li J, Yin Y. Chemically crosslinked alginate porous microcarriers modified with bioactive molecule for expansion of human hepatocellular carcinoma cells. J.Biomed.Mater. Res.B.Appl.Biomater. 102, 1648-1658 (2014).

Li H, Frith J, Cooper-White JJ. Modulation of Stem Cell Adhesion and Morphology via Facile Control over Surface Presentation of Cell Adhesion Molecules. Biomacromolecules 15, 43-52 (2014).

Li L, Davidovich AE, Schloss JM, et al. Neural lineage differentiation of embryonic stem cells within alginate microbeads. Biomaterials 32, 4489-4497 (2011).

Li X, Liu T, Song K, et al. Culture of neural stem cells in calcium alginate beads. Biotechnol.Prog. 22, 1683-1689 (2006).

Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 210, 908-910 (1980).

Liu H, Lu K, Macary PA, et al. Soluble molecules are key in maintaining the immunomodulatory activity of murine mesenchymal stromal cells. J.Cell.Sci. 125, 200-208 (2012).

Liu ZC, Chang TM. Coencapsulation of hepatocytes and bone marrow stem cells: in vitro conversion of ammonia and in vivo lowering of bilirubin in hyperbilirubemia Gunn rats. Int.J.Artif.Organs 26, 491-497 (2003).

Lubiatowski P, Kruczynski J, Gradys A, Trzeciak T, Jaroszewski J. Articular cartilage repair by means of biodegradable scaffolds. Transplant.Proc. 38, 320-322 (2006).

Luca G, Fallarino F, Calvitti M, et al. Xenograft of microencapsulated sertoli cells reverses T1DM in NOD mice by inducing neogenesis of beta-cells. Transplantation 90, 1352-1357 (2010).

Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292, 1389-1394 (2001).

Ma HL, Hung SC, Lin SY, Chen YL, Lo WH. Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. J.Biomed.Mater.Res.A. 64, 273-281 (2003).

Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. Cell Death Differ. 21, 216-225 (2014).

Machida-Sano I, Ogawa S, Ueda H, Kimura Y, Satoh N, Namiki H. Effects of composition of iron-cross-linked alginate hydrogels for cultivation of human dermal fibroblasts. Int.J.Biomater. 2012, 820513 (2012).

Maehr R, Chen S, Snitow M, et al. Generation of pluripotent stem cells from patients with type 1

diabetes. Proc.Natl.Acad.Sci.U.S.A. 106, 15768-15773 (2009).

Maguire T, Novik E, Schloss R, Yarmush M. Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes. Biotechnol.Bioeng. 93, 581-591 (2006).

Mallett AG, Korbutt GS. Alginate modification improves long-term survival and function of transplanted encapsulated islets. Tissue Eng.Part A. 15, 1301-1309 (2009).

Markusen JF, Mason C, Hull DA, et al. Behavior of adult human mesenchymal stem cells entrapped in alginate-GRGDY beads. Tissue Eng. 12, 821-830 (2006).

Martinsen A, Skjak-Braek G, Smidsrod O. Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol.Bioeng. 33, 79-89 (1989).

Matyash M, Despang F, Mandal R, Fiore D, Gelinsky M, Ikonomidou C. Novel soft alginate hydrogel strongly supports neurite growth and protects neurons against oxidative stress. Tissue Eng.Part A. 18, 55-66 (2012).

Mazzitelli S, Luca G, Mancuso F, et al. Production and characterization of engineered alginate-based microparticles containing ECM powder for cell/tissue engineering applications. Acta Biomater. 7, 1050-1062 (2011).

McQuilling JP, Arenas-Herrera J, Childers C, et al. New alginate microcapsule system for angiogenic protein delivery and immunoisolation of islets for transplantation in the rat omentum pouch. Transplant.Proc. 43, 3262-3264 (2011).

McMahon JM, Conroy S, Lyons M, et al. Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral vectors. Stem Cells Dev. 15, 87-96 (2006).

Mello MA, Tuan RS. Effects of TGF-beta1 and triiodothyronine on cartilage maturation: in vitro analysis using long-term high-density micromass cultures of chick embryonic limb mesenchymal cells. J.Orthop.Res. 24, 2095-2105 (2006).

Mobasheri A, Csaki C, Clutterbuck AL, Rahmanzadeh M, Shakibaei M. Mesenchymal stem cells in connective tissue engineering and regenerative medicine: applications in cartilage repair and osteoarthritis therapy. Histol.Histopathol. 24, 347-366 (2009).

Morch YA, Donati I, Strand BL, Skjak-Braek G. Effect of Ca2+, Ba2+, and Sr2+ on alginate microbeads. Biomacromolecules 7, 1471-1480 (2006).

Moreau JL, Xu HH. Mesenchymal stem cell proliferation and differentiation on an injectable calcium phosphate-chitosan composite scaffold. Biomaterials 30, 2675-2682 (2009).

Munirah S, Samsudin OC, Aminuddin BS, Ruszymah BH. Expansion of human articular chondrocytes and formation of tissue-engineered cartilage: A step towards exploring a potential use of matrix-induced cell therapy. Tissue Cell (2010).

Murua A, de Castro M, Orive G, Hernandez RM, Pedraz JL. In vitro characterization and in vivo functionality of erythropoietin-secreting cells immobilized in alginate-poly-L-lysine-alginate microcapsules. Biomacromolecules 8, 3302-3307 (2007).

Murua A, Herran E, Orive G, et al. Design of a composite drug delivery system to prolong functionality of cell-based scaffolds. Int.J.Pharm. 407, 142-150 (2011).

Nafea EH, Marson A, Poole-Warren LA, Martens PJ. Immunoisolating semi-permeable membranes for cell encapsulation: focus on hydrogels. J.Control.Release 154, 110-122 (2011).

Naftanel MA, Harlan DM. Pancreatic islet transplantation. PLoS Med. 1, e58; quiz e75 (2004).

Nair A, Shen J, Lotfi P, Ko CY, Zhang CC, Tang L. Biomaterial implants mediate autologous stem cell recruitment in mice. Acta Biomater. 7, 3887-3895 (2011).

Nayan M, Paul A, Chen G, Chiu RC, Prakash S, Shum-Tim D. Superior therapeutic potential of young bone marrow mesenchymal stem cells by direct intramyocardial delivery in aged recipients with acute myocardial infarction: in vitro and in vivo investigation. J.Tissue Eng. 2011, 741213 (2011).

Neff JA, Tresco PA, Caldwell KD. Surface modification for controlled studies of cell-ligand interactions. Biomaterials 20, 2377-2393 (1999).

Nejadnik H, Hui JH, Feng Choong EP, Tai BC, Lee EH. Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study. Am.J.Sports Med. 38, 1110-1116 (2010).

Ngoc PK, Phuc PV, Nhung TH, Thuy DT, Nguyet NT. Improving the efficacy of type 1 diabetes therapy by transplantation of immunoisolated insulin-producing cells. Hum.Cell 24, 86-95 (2011).

Orive G, Anitua E, Pedraz JL, Emerich DF. Biomaterials for promoting brain protection, repair and regeneration. Nat.Rev.Neurosci. 10, 682-692 (2009).

Orive G, De Castro M, Kong HJ, et al. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. J.Control.Release 135, 203-210 (2009).

Orive G, Hernandez RM, Gascon AR, Igartua M, Pedraz JL. Survival of different cell lines in alginate-agarose microcapsules. Eur.J.Pharm.Sci. 18, 23-30 (2003).

Orive G, Santos E, Pedraz JL, Hernandez RM. Application of cell encapsulation for controlled

delivery of biological therapeutics. Adv.Drug Deliv.Rev. (2013).

Orive G, Tam SK, Pedraz JL, Halle JP. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. Biomaterials 27, 3691-3700 (2006).

Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY. Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat.Biotechnol. 20, 1103-1110 (2002).

Park H, Temenoff JS, Holland TA, Tabata Y, Mikos AG. Delivery of TGF-beta1 and chondrocytes via injectable, biodegradable hydrogels for cartilage tissue engineering applications. Biomaterials 26, 7095-7103 (2005).

Park JS, Shim MS, Shim SH, et al. Chondrogenic potential of stem cells derived from amniotic fluid, adipose tissue, or bone marrow encapsulated in fibrin gels containing TGF-beta3. Biomaterials 32, 8139-8149 (2011).

Patil AS, Sable RB, Kothari RM. An update on transforming growth factor-beta (TGF-beta): sources, types, functions and clinical applicability for cartilage/bone healing. J.Cell.Physiol. 226, 3094-3103 (2011).

Paul A, Nayan M, Khan AA, Shum-Tim D, Prakash S. Angiopoietin-1-expressing adipose stem cells genetically modified with baculovirus nanocomplex: investigation in rat heart with acute infarction. Int.J.Nanomedicine 7, 663-682 (2012).

Pawar SN, Edgar KJ. Alginate derivatization: A review of chemistry, properties and applications. Biomaterials 33, 3279-3305 (2012).

Perrier E, Ronziere MC, Bareille R, Pinzano A, Mallein-Gerin F, Freyria AM. Analysis of collagen expression during chondrogenic induction of human bone marrow mesenchymal stem cells. Biotechnol.Lett. 33, 2091-2101 (2011).

Pierschbacher MD, Ruoslahti E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. J.Biol.Chem. 262, 17294-17298 (1987).

Pili D, Tranquilli Leali P. Biomaterials and bone. Aging Clin.Exp.Res. 23, 74-75 (2011).

Pleumeekers MM, Nimeskern L, Koevoet WL, et al. The in vitro and in vivo capacity of culture-expanded human cells from several sources encapsulated in alginate to form cartilage. Eur.Cell. Mater. 27, 264-80; discussion 278-80 (2014).

Plopper GE, McNamee HP, Dike LE, Bojanowski K, Ingber DE. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol.Biol.Cell 6, 1349-1365 (1995).

Ponce S, Orive G, Hernandez R, et al. Chemistry and the biological response against immunoisolating alginate-polycation capsules of different composition. Biomaterials 27, 4831-4839 (2006).

Popa EG, Gomes ME, Reis RL. Cell delivery systems using alginate--carrageenan hydrogel beads and fibers for regenerative medicine applications. Biomacromolecules 12, 3952-3961 (2011).

Prang P, Muller R, Eljaouhari A, et al. The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. Biomaterials 27, 3560-3569 (2006).

Razavi S, Razavi MR, Kheirollahi-Kouhestani M, Mardani M, Mostafavi FS. Co-culture with neurotrophic factor secreting cells induced from adipose-derived stem cells: promotes neurogenic differentiation. Biochem.Biophys.Res.Commun. 440, 381-387 (2013).

Re'em T, Tsur-Gang O, Cohen S. The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGFbeta1-induced chondrogenesis of human mesenchymal stem cells. Biomaterials 31, 6746-6755 (2010).

Rege TA, Fears CY, Gladson CL. Endogenous inhibitors of angiogenesis in malignant gliomas: nature's antiangiogenic therapy. Neuro Oncol. 7, 106-121 (2005).

Remminghorst U, Rehm BH. Bacterial alginates: from biosynthesis to applications. Biotechnol.Lett. 28, 1701-1712 (2006).

Richardson T, Kumta PN, Banerjee I. Alginate encapsulation of human embryonic stem cells to enhance directed differentiation to pancreatic islet-like cells. Tissue Eng.Part A. 20, 3198-3211 (2014).

Roach P, Farrar D, Perry CC. Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. J.Am.Chem.Soc. 128, 3939-3945 (2006).

Rowley JA, Madlambayan G, Mooney DJ. Alginate hydrogels as synthetic extracellular matrix materials. Biomaterials 20, 45-53 (1999).

Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. J.Biomed. Mater.Res. 60, 217-223 (2002).

Ruoslahti E. RGD and other recognition sequences for integrins. Annu.Rev.Cell Dev.Biol. 12, 697-715 (1996).

Saenz Del Burgo L, Compte M, Aceves M, et al. Microencapsulation of therapeutic bispecific antibodies producing cells: immunotherapeutic organoids for cancer management. J.Drug Target. 23, 170-179 (2015).

Safley SA, Cui H, Cauffiel SM, Xu BY, Wright JR,Jr, Weber CJ. Encapsulated piscine (tilapia) islets for

diabetes therapy: studies in diabetic NOD and NOD-SCID mice. Xenotransplantation 21, 127-139 (2014).

Salinas CN, Anseth KS. The influence of the RGD peptide motif and its contextual presentation in PEG gels on human mesenchymal stem cell viability. J.Tissue Eng.Regen.Med. 2, 296-304 (2008).

Salinas CN, Anseth KS. Decorin moieties tethered into PEG networks induce chondrogenesis of human mesenchymal stem cells. J.Biomed.Mater.Res.A. 90, 456-464 (2009).

Salinas CN, Anseth KS. Mesenchymal stem cells for craniofacial tissue regeneration: designing hydrogel delivery vehicles. J.Dent.Res. 88, 681-692 (2009).

Santos E, Garate A, Pedraz JL, Orive G, Hernandez RM. The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells: In vitro and in vivo direct comparative study. J.Biomed.Mater.Res.A. (2013).

Santos E, Hernandez RM, Pedraz JL, Orive G. Novel advances in the design of three-dimensional bio-scaffolds to control cell fate: translation from 2D to 3D. Trends Biotechnol. 30, 331-341 (2012).

Santos E, Pedraz JL, Hernandez RM, Orive G. Therapeutic cell encapsulation: ten steps towards clinical translation. J.Control.Release 170, 1-14 (2013).

Santos E, Zarate J, Orive G, Hernandez RM, Pedraz JL. Biomaterials in cell microencapsulation. Adv. Exp.Med.Biol. 670, 5-21 (2010).

Sawyer AA, Hennessy KM, Bellis SL. Regulation of mesenchymal stem cell attachment and spreading on hydroxyapatite by RGD peptides and adsorbed serum proteins. Biomaterials 26, 1467-1475 (2005).

Sawyer AA, Hennessy KM, Bellis SL. The effect of adsorbed serum proteins, RGD and proteoglycan-binding peptides on the adhesion of mesenchymal stem cells to hydroxyapatite. Biomaterials 28, 383-392 (2007).

Sayyar B, Dodd M, Wen J, et al. Encapsulation of factor IX-engineered mesenchymal stem cells in fibrinogen-alginate microcapsules enhances their viability and transgene secretion. J.Tissue Eng. 3, 2041731412462018 (2012).

Sayyar B, Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Cell-matrix Interactions of Factor IX (FIX)-engineered human mesenchymal stromal cells encapsulated in RGD-alginate vs. Fibrinogen-alginate microcapsules. Artif.Cells Nanomed Biotechnol. (2013).

Sayyar B, Dodd M, Marquez-Curtis L, Janowska-Wieczorek A, Hortelano G. Fibronectin-Alginate microcapsules improve cell viability and protein secretion of encapsulated Factor IX-engineered

human mesenchymal stromal cells. Artif.Cells Nanomed Biotechnol. (2014).

Seidlits SK, Khaing ZZ, Petersen RR, et al. The effects of hyaluronic acid hydrogels with tunable mechanical properties on neural progenitor cell differentiation. Biomaterials 31, 3930-3940 (2010).

Seo SJ, Akaike T, Choi YJ, Shirakawa M, Kang IK, Cho CS. Alginate microcapsules prepared with xyloglucan as a synthetic extracellular matrix for hepatocyte attachment. Biomaterials 26, 3607-3615 (2005).

Shah K. Mesenchymal stem cells engineered for cancer therapy. Adv.Drug Deliv.Rev. (2011).

Shakibaei M, De Souza P. Differentiation of mesenchymal limb bud cells to chondrocytes in alginate beads. Cell Biol.Int. 21, 75-86 (1997).

Shin H, Jo S, Mikos AG. Biomimetic materials for tissue engineering. Biomaterials 24, 4353-4364 (2003).

Sidhu K, Kim J, Chayosumrit M, Dean S, Sachdev P. Alginate microcapsule as a 3D platform for propagation and differentiation of human embryonic stem cells (hESC) to different lineages. J.Vis. Exp. (61). pii: 3608. doi, 10.3791/3608 (2012).

Sinha RK, Tuan RS. Regulation of human osteoblast integrin expression by orthopedic implant materials. Bone 18, 451-457 (1996).

Solorio LD, Vieregge EL, Dhami CD, Dang PN, Alsberg E. Engineered cartilage via self-assembled hMSC sheets with incorporated biodegradable gelatin microspheres releasing transforming growth factor-beta1. J.Control.Release (2011).

Spradling Allan, Drummond-Barbosa Daniela, Kai Toshie. Stem cells find their niche.

Springer ML, Hortelano G, Bouley DM, Wong J, Kraft PE, Blau HM. Induction of angiogenesis by implantation of encapsulated primary myoblasts expressing vascular endothelial growth factor. J.Gene Med. 2, 279-288 (2000).

Stupack DG, Cheresh DA. Get a ligand, get a life: integrins, signaling and cell survival. J.Cell.Sci. 115, 3729-3738 (2002).

Stupack DG, Cheresh DA. Apoptotic cues from the extracellular matrix: regulators of angiogenesis. Oncogene 22, 9022-9029 (2003).

Sumi S. Regenerative medicine for insulin deficiency: creation of pancreatic islets and bioartificial pancreas. J.Hepatobiliary.Pancreat.Sci. 18, 6-12 (2011).

Tam SK, de Haan BJ, Faas MM, Halle JP, Yahia L, de Vos P. Adsorption of human immunoglobulin to implantable alginate-poly-L-lysine microcapsules: effect of microcapsule composition. J.Biomed. Mater.Res.A. 89, 609-615 (2009).

Tang J, Xie Q, Pan G, Wang J, Wang M. Mesenchymal stem cells participate in angiogenesis and improve heart function in rat model of myocardial ischemia with reperfusion. Eur.J.Cardiothorac. Surg. 30, 353-361 (2006).

Tasso R, Gaetani M, Molino E, et al. The role of bFGF on the ability of MSC to activate endogenous regenerative mechanisms in an ectopic bone formation model. Biomaterials 33, 2086-2096 (2012).

Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. Am.J.Physiol.Renal Physiol. 292, F1626-35 (2007).

Tompkins RG, Carter EA, Carlson JD, Yarmush ML. Enzymatic function of alginate immobilized rat hepatocytes. Biotechnol.Bioeng. 31, 11-18 (1988).

Trouche E, Girod Fullana S, Mias C, et al. Evaluation of alginate microspheres for mesenchymal stem cell engraftment on solid organ. Cell Transplant. 19, 1623-1633 (2010).

Tsai S, Jeng M, Tsay R, Wang Y. Gel beads composed of collagen reconstituted in alginate. Biotech Tech 12, 21-23 (1998).

Tuch BE, Hughes TC, Evans MD. Encapsulated pancreatic progenitors derived from human embryonic stem cells as a therapy for insulin-dependent diabetes. Diabetes Metab.Res.Rev. 27, 928-932 (2011).

Tuli R, Tuli S, Nandi S, et al. Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. J.Biol.Chem. 278, 41227-41236 (2003).

Valiani A, Hashemibeni B, Esfandiary E, Ansar MM, Kazemi M, Esmaeili N. Study of carbon nano-tubes effects on the chondrogenesis of human adipose derived stem cells in alginate scaffold. Int.J.Prev.Med. 5, 825-834 (2014).

Wang N, Adams G, Buttery L, Falcone FH, Stolnik S. Alginate encapsulation technology supports embryonic stem cells differentiation into insulin-producing cells. J.Biotechnol. 144, 304-312 (2009).

Wang X, Yan C, Ye K, He Y, Li Z, Ding J. Effect of RGD nanospacing on differentiation of stem cells. Biomaterials 34, 2865-2874 (2013).

Wang X, Ye K, Li Z, Yan C, Ding J. Adhesion, proliferation, and differentiation of mesenchymal stem

cells on RGD nanopatterns of varied nanospacings. Organogenesis 9, (2013).

White SA, Shaw JA, Sutherland DE. Pancreas transplantation. Lancet 373, 1808-1817 (2009).

Williams DF. The role of short synthetic adhesion peptides in regenerative medicine; the debate. Biomaterials 32, 4195-4197 (2011).

Woods A, Couchman JR, Johansson S, Hook M. Adhesion and cytoskeletal organisation of fibroblasts in response to fibronectin fragments. EMBO J. 5, 665-670 (1986).

Wright EJ, Farrell KA, Malik N, et al. Encapsulated glucagon-like peptide-1-producing mesenchymal stem cells have a beneficial effect on failing pig hearts. Stem Cells Transl.Med. 1, 759-769 (2012).

Xiang M, Lin Y, He G, et al. Correlation between biological activity and binding energy in systems of integrin with cyclic RGD-containing binders: a QM/MM molecular dynamics study. J.Mol.Model. 18, 4917-4927 (2012).

Xiang MX, He AN, Wang JA, Gui C. Protective paracrine effect of mesenchymal stem cells on cardiomyocytes. J.Zhejiang Univ.Sci.B. 10, 619-624 (2009).

Yasuhara T, Shingo T, Muraoka K, et al. Neurorescue effects of VEGF on a rat model of Parkinson's disease. Brain Res. 1053, 10-18 (2005).

Yu J, Du KT, Fang Q, et al. The use of human mesenchymal stem cells encapsulated in RGD modified alginate microspheres in the repair of myocardial infarction in the rat. Biomaterials 31, 7012-7020 (2010).

Zhao L, Weir MD, Xu HH. An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. Biomaterials 31, 6502-6510 (2010).

Zhou B, Tsaknakis G, Coldwell KE, et al. A novel function for the haemopoietic supportive murine bone marrow MS-5 mesenchymal stromal cell line in promoting human vasculogenesis and angiogenesis. Br.J.Haematol. (2012).

Zhou H, Xu HH. The fast release of stem cells from alginate-fibrin microbeads in injectable scaffolds for bone tissue engineering. Biomaterials 32, 7503-7513 (2011).

Zhou M, Li P, Tan L, Qu S, Ying QL, Song H. Differentiation of mouse embryonic stem cells into hepatocytes induced by a combination of cytokines and sodium butyrate. J.Cell.Biochem. 109, 606-614 (2010).

Zhu Y, Liu T, Song K, Ning R, Ma X, Cui Z. ADSCs differentiated into cardiomyocytes in cardiac microenvironment. Mol.Cell.Biochem. 324, 117-129 (2009).

Zimmermann H, Zimmermann D, Reuss R, et al. Towards a medically approved technology for alginate-based microcapsules allowing long-term immunoisolated transplantation. J.Mater.Sci. Mater.Med. 16, 491-501 (2005).