

Full Length Research Paper

3-D osteoblast culture for biomaterials testing

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Micromass cell cultures, osteomicrospheres, were formed by aggregation of primary osteoblasts. Cell differentiation during sphere formation and the integrity of the osteomicrospheres was evaluated by analyzing the immunohistochemical expression of osteonectin, osteocalcin, and collagen type I. Transmission electron microscopy facilitated the proof of the tissue-like microstructure inside the osteomicrospheres and the arrangement of collagen fibers similar to early stages of natural bone formation. The *in vivo* situation of osteomicrospheres transferred to host tissue was simulated by embedding mature osteomicrospheres in a fibrin matrix. Additionally, cell spreading out of the compartments was investigated by electron microscopy of microspheres cultivated on the surface of three-dimensional fiber-like scaffolds. Concluding, the properties of the osteomicrospheres represented in this work demonstrate the potential as a tissue-specific *in vitro* test method to replace early small animal tests in the future.

Key words: Biomaterials testing, micromass culture, osteoblast, osteomicrospheres, ultrastructure.

INTRODUCTION

Tissue equivalent, three-dimensional cell agglomerates so-called micromass cultures show similar cell proliferation and differentiation like cells *in vivo*. In different studies micro mass cultures of osteoblasts, osteoblast-like cells and other cell types like stem cells (Langenbach et al., 2011, Zhang et al., 2010) were investigated intensively which affirms this tissue equivalent cell behavior.

New therapeutic approaches for the regeneration of bone require comprehensive understanding of the cellular and tissue level mechanisms that underlie bone development and bone healing. To improve bone tissue engineering strategies it seems suitable to mimic the native developmental mechanisms of bone growth and differentiation. Therefore, new insights are expected from 3-D spheroid cell culture systems (Bates et al., 2000). Numerous *in vitro* studies were performed to evaluate the cell behavior in various three-dimensional artificial scaffold materials (Meyer et al., 2004; Wiesmann et al.,

2004). Spheroids are developed from cells by a variety of methods: i) plating cells in gyratory shakers, roller flasks, or spinner flasks that continuously rotate preventing cellular adherence to the walls; ii) coating tissue culture surfaces with non adhesive substance (Iwasaki et al., 2009); iii) using the hanging drop culture method; iv) by centrifuge compression of cells and v) by the use of appropriate growth factors.

Increasing evidence has clearly stated that changes in cell shape as present under 3-D culture conditions can actually affect the cell fate. Cell morphology was shown to influence proliferation (Chen et al., 1997, Rossi et al., 2005), differentiation and gene expression (Rossi et al., 2005). The accumulation of experimental results indicates that in the absence of an anchoring material, intercellular adhesion may provide signals which promote cell activity. The aim of the present study was to generate 3-D micro-mass tissues to characterize tissue formation by histochemical, immunohistochemical, and microscopically techniques. Emphasis is given to the feature of mineral formation in the newly formed extracellular matrix (ECM). Furthermore the potential of the osteomicrospheres as an *in vitro* test system was evaluated.

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MATERIALS AND METHODS

Harvesting of osteoblast-like cells and sphere formation

The periosteal layer of calf metacarpus was aseptically stripped off the bone, cut into small pieces and plated into polystyrene culture dishes with their osteogenic side down-facing. High growth enhancement medium (HGEM)(MP Biomedicals GmbH) supplemented with 10 % fetal calf serum, 250 µg/ml amphotericin B, 10000 IU/ml penicillin, 10000 IU/ml streptomycin and 200 mmol/l L-glutamine (Biochrom KG seromed) was used for cultivation and was replaced once a week. Incubation was carried out at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Bovine primary osteoblast-like cells were harvested after three weeks by collagenase incubation (Biochrom KG seromed), counted using a coulter counting system (CASY 1 Model TT, Schaefer System GmbH) and transferred to a non-attachment environment. Therefore agarose (Biozym Scientific GmbH) coated chambers of 96-well plates were prepared by applying 50 µl of a warm mixture of 20 mg/ml agarose (Biozym Scientific GmbH) in DMEM (Biochrom)/HGEM per well. A population of 200,000 cells per well was seeded and incubated in HGEM as mentioned above. The medium was changed twice weekly. After seven days of cultivation, osteomicrospheres were analyzed or used for biomaterial testing.

Cultivating osteomicrospheres on biological environments

In order to evaluate the potency of the osteomicrospheres to interact with biological environments, seven days old microspheres were aspirated with a pipette and transferred to be cultured in a fibrin matrix or on a fibroid silica-gel biomaterial scaffold (Bayer Innovation GmbH). The sphere-seeded materials were evaluated after three days by histological techniques or scanning electron microscope (SEM), respectively. As a reference, cell microspheres were cultivated on glass chamber slides (Nunc) and evaluated by light microscopy after seven and 28 days after hematoxyline and eosin (H&E) staining (Merck).

Histological and immunohistological procedures

For histology, evaluation of cell viability and differentiation steps up to cell mineralization, the osteomicrospheres were harvested after three, seven, 14, and 28 days, respectively. The osteomicrospheres were fixed with 4% formalin, embedded in paraffin, sectioned by a microtome (Ultracut S, Reichert), and stained with H&E (Merck). Mouse monoclonal primary antibodies against collagen type I (Abcam) 1:100 diluted, osteocalcin (TaKaRa) 1:100 diluted, and a rabbit polyclonal antibody for osteonectin (Chemicon International) 1:50 diluted in 1 % BSA in PBS were used for immunohistochemistry. Secondary antibodies used were anti-mouse (Dako K-4001) and anti-rabbit antibodies (Dako K-4002). Sections were deparaffinized by immersing the samples twice in xylene for 5 min, followed by gradual dehydration of the sections through immersion in ethanol. Finally, sections were washed with distilled water.

Transmission electron microscopy (TEM)

Samples were fixed in 0.1 M phosphate buffer containing 2.5% glutaraldehyde (Sigma-Aldrich) dehydrated in an ascending series of ethanol and embedded in araldite resin (Plano). Ultrathin sections of 70 to 90 nm were cut (Leica RML 155) and stained for 30 min with 2% uranyl acetate (Plano) followed by incubation in Reynolds's lead citrate (Merck) for 3 min. Sections were examined with a Zeiss TEM 902 at an acceleration voltage of 80 kV.

Scanning electron microscopy (SEM)

Osteomicrospheres cultivated for three days on the fibroid biomaterial scaffold were fixed with glutaraldehyde (Sigma), followed by dehydration in graded series of ethanol, and chemical drying through the iterative transfer into hexamethyldisilazane (HMDS; Fluka). The samples were fixed on SEM stubs and sputtered with platinum.

RESULTS

After seven days of micromass formation, the H&E staining of paraffin sections (Figure 1a) revealed the integrity of highly aggregated osteoblast-like cells which formed round-shaped osteomicrospheres. Sphere diameters varied from 250 to 300 µm. Cell nuclei were visible in all regions of the microspheres. Where the inner core appeared less organized, the outer regions were even more oriented to form a dense layer of elongated cells on the surface of the osteomicrospheres (Figure 1a). The sphere's surface was built up from three to four cell layers superimposed by each other. Some regions of lower cell density appeared as bright spots distributed evenly throughout the sphere. Intracellular space occurred as thin layers containing extracellular matrix.

The *in vitro* immunoeexpression of extracellular matrix proteins demonstrated an increased level of collagen type I during 28 days of sphere formation and cultivation (Figure 1b). After three days, first spots became visible and developed a heterogeneous pattern until day seven. After 14 days the distribution of collagen type I was homogeneous over the whole cross-section of the osteomicrospheres. The total expression of the non-collagenous matrix protein osteonectin varied slightly during cultivation time. Obviously, the distribution of the protein was enhanced in the core region of the microspheres compared to the outer regions. The expression of osteocalcin was evenly after 3 days. However, the development to a core region of high expression level and an outer shell of lower osteocalcin expression level lasted until day seven. Microsphere maturation led to increased OC expression throughout the whole sphere. Cell differentiation and outgrowth over 28 days of cultivation varied activity and viability of the cells in the all regions of the spheres.

Proceeding outgrowth of single cells during cultivation caused decreasing sphere sizes, particularly starting from day seven. This process is also visualized in Figures 3b and c, showing cells outgrowing from a sphere and migrating radially to form a layer after seven days of cultivation on glass. Ultrastructural analysis carried out using transmission electron microscopy confirmed the sphere's shell to be composed of flattened, thin and elongated osteoblasts containing few organelles. Osteoblasts located in the core of the osteomicrospheres did not exhibit a directed orientation but exhibited large oval shaped nuclei and an active-state organelle system: extended rough endoplasmic reticulum, mitochondria,

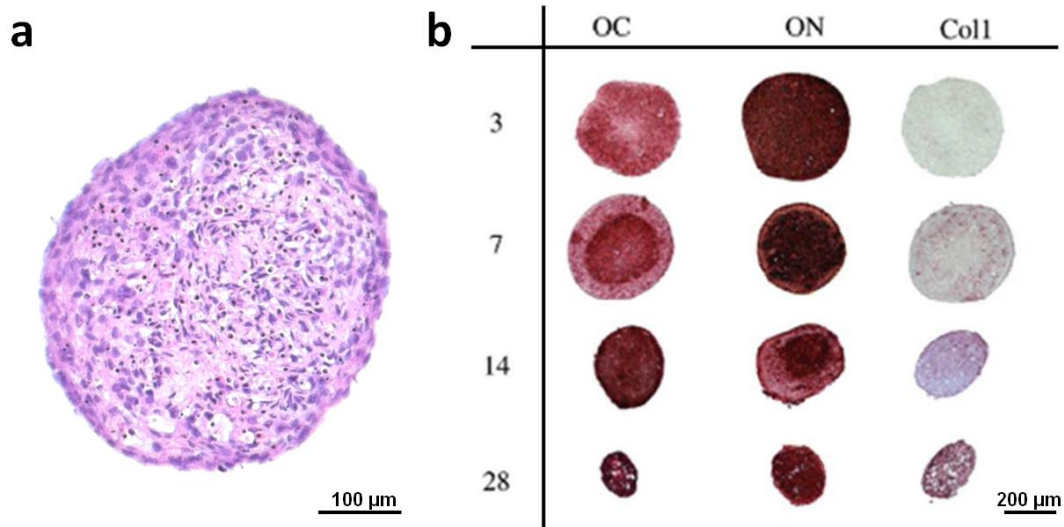


Figure 1. a) H&E staining of a paraffin section of an osteomicrosphere formed after seven days of cultivation with elongated cells forming the sphere's shell in the outer region. b) Micrographs of osteomicrospheres formed after 3, 7, 14, and 28 days of micromass cultivation stained for osteocalcin (OC), osteonectin (ON), and collagen I (Col I), respectively.

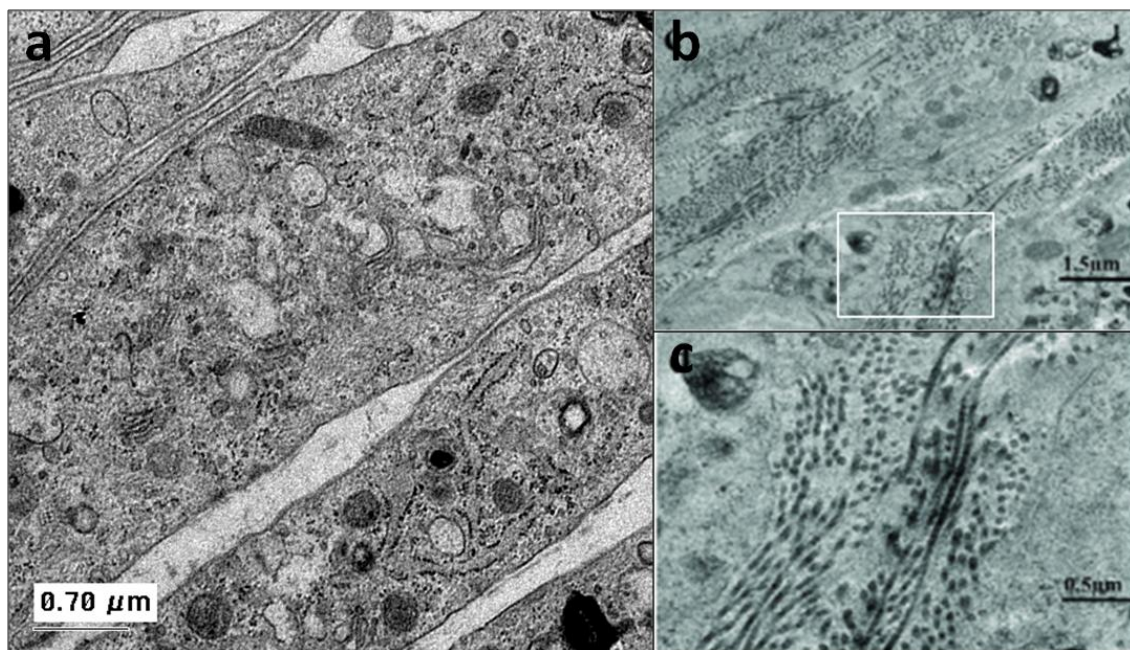


Figure 2. TEM image showing a) elongated cells b) developed, organized collagen bundles in a 21 day old osteomicrosphere and c) magnification of b).

secretory vesicles, polysomes, and gap junctions, all these features proving high metabolic activity (Figure 2).

The occurrence of extracellular matrix formation varied according to the location in the sphere, being concentrated to the core and less abundant in the outer layers. Additionally, extracellular matrix was less

prominent in younger osteomicrospheres and more apparent in older microspheres. Continuous collagen fiber formation in the extracellular matrix of the microspheres was demonstrated throughout the entire cultivation time. Orientation of the fibrils was similar to the *in vivo* situation (Figures 2b and c). Directly after transfer

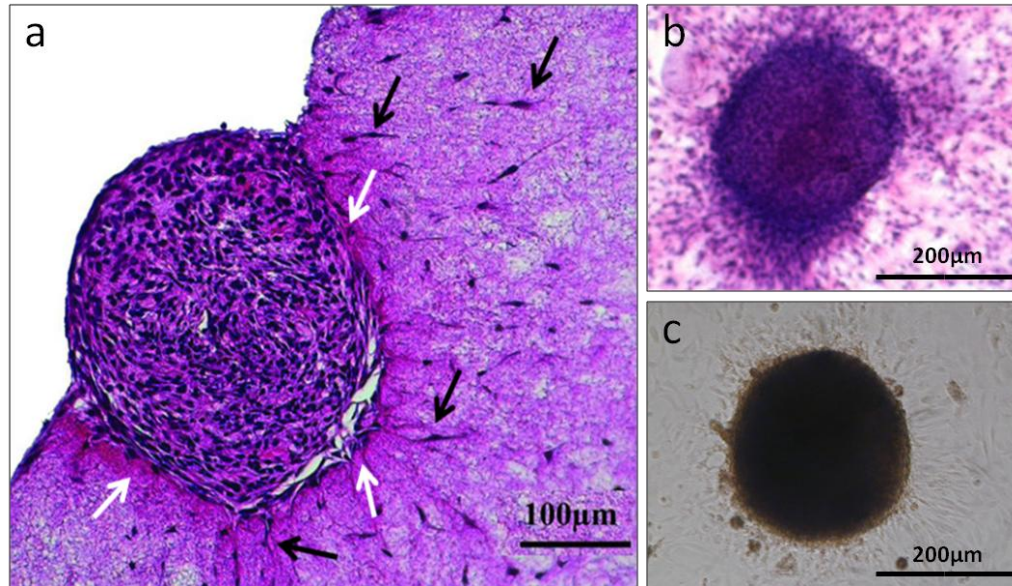


Figure 3. Imaging of cell migration of 7 days old osteomicrosphere after 3 days of incubation on fibrin membrane after H&E-staining (a). Several cells spread out of the sphere to the interface (white arrows) and infiltrated the artificial matrix (black arrows). Micromass culture after seven days of cultivation on glass H&E-stained (b) and without staining (c).

to the fibrin, the osteomicrospheres attached to the artificial matrix. After seven days, the cultivation of osteomicrospheres in the matrix led to an intimate contact of the sphere's cells with the fibrin surface. Migration of osteoblasts on the interface (white arrows in Figure 3a) as well as into the fibrin matrix (black arrows Figure 3a) was observed histologically.

Figures 3b and c show the behavior of the agglomerated cells on untreated glass slides. The tendency of the individual cells to grow out of the organized osteomicrospheres and to colonize the surrounding area or substrate is obvious. Subsequently, osteomicrospheres were assessed in contact with an artificial environment which facilitates the observation of three-dimensional sphere attachment as well as cell spreading. Therefore, seven days old microspheres were transferred to a fiber-like scaffold instead of a conventional foam-like scaffold and were evaluated morphologically using SEM (Figure 4). After three days of cultivation, osteomicrospheres attached to the silica fiber scaffold, showed the attempt to build up larger tissue-like formations by forming agglomerates of several osteomicrospheres (Figure 4a) and enveloped single fibers (Figure 4b). This resulted in covering of the fiber material by outgrown cells and corresponding extracellular matrix.

DISCUSSION

Edwards and Mason (2006) postulated that the use of passive three-dimensional scaffolds, inductive strategies

in which additional growth factors are incorporated into scaffold/matrix-systems to modify cell behavior, or strategies to form vital constructs of cells are important future strategies for tissue regeneration. The present study focuses on the third point.

The use of micromass cultures is a recently elaborated technique in bone biology since basic principles of cell and tissue development in three-dimensional space can be evaluated and basic tissue engineering techniques can be approached. Recent studies focus on two aspects: to initiate and investigate the basic principles of cellular self-assembly, and the scaffold-free formation of three-dimensional tissue (Battistelli et al., 2005, Tare et al., 2005). In the literature, different methods are reported which facilitate the formation of spherical-shaped micromass cultures (Handschel et al., 2007, Kale et al., 2000, Tortelli and Cancedda, 2009). These should mimic the targeted tissues; resemble their organization, their mechanical properties, and their physiological response to different stimuli (Tortelli and Cancedda, 2009).

In the present study osteomicrospheres were derived from conventional monolayers of primary osteoblast-like cells and showed bone tissue-like differentiation. The scaffold-free three-dimensional tissue was built up from differentiated osteoblast-like cells in high density and showed tissue-like behavior also during cultivation on artificial biomaterials.

Concerning the usage of cell spheroids, different studies constituted that three-dimensional cultures exert higher proliferation rates than cells cultured in two-dimensional systems. Furthermore their cell-specific differentiation and tissue-like migration is more distinct

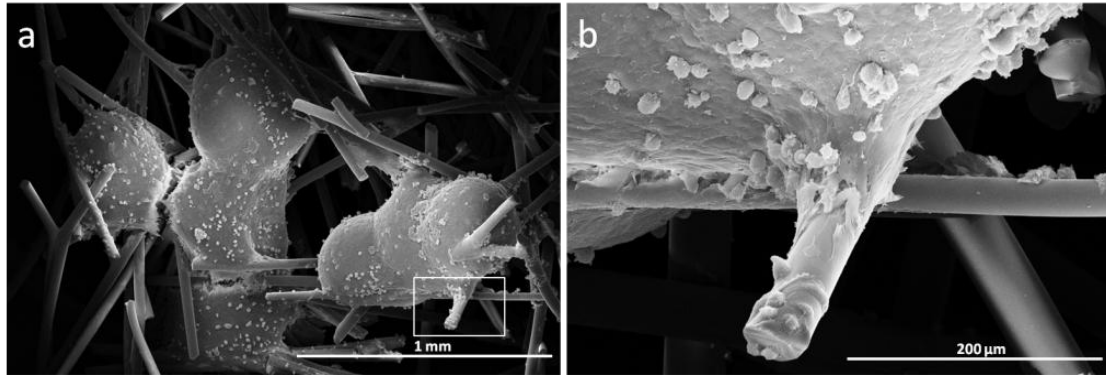


Figure 4. SEM overview a) of seven days old osteomicrospheres after three days of cultivation on a silica fiber scaffold. The osteomicrospheres showed strong attachment to the underlying fiber material. b) Outgrowing sphere cells covering the fiber surface.

(Abbott, 2003; Cukierman et al., 2001; Handschel et al., 2007). Moreover, the cells are flexible to change their shape and are able to react in a more physiological way when they are cultured in three-dimensional spheres; reasons are cell-matrix and cell-cell contacts supporting specific cell signals and migration of the cells (Handschel et al., 2011, Sivaraman et al., 2005; Weaver et al., 1997; Yamada and Geiger, 1997).

The micromass culture technique represents an alternative for substituting artificial scaffolds and a potent method for biomaterials testing. In contrast to two-dimensional monolayers, the three-dimensional culture technique shows more similarities to tissues under *in-vivo* conditions (Handschel et al., 2007). It shall be deemed to be proven that a lot of cellular functions, e.g. differentiation, migration, and proliferation, rely on intact cell-matrix and cell-cell interactions and an intimate attachment to components of the extracellular matrix. In the three-dimensional spheres, the cells can interact with each other and maintain these essential interactions (Boudreau and Jones, 1999; Cukierman et al., 2001; Yamada and Geiger, 1997).

These results correspond to findings concerning the spheroid behavior presented by Gerber et al. (2005). They also highlight faster differentiation and mineralization of micromass-cultures compared to conventional two-dimensional culture methods of the same cell types. Whereas the formation of osteomicrospheres is seen regularly in the non-attachment chambers, we observed a limited control over shape or size of the produced tissue. This may be based on insufficient non-attachment behavior of agarose, being not resistant to the inevitable precipitation of medium proteins forming a new interface layer. An increased cell detachment from the sphere and attachment onto the agarose-protein layer is observed over time. During the cultivation of about 28 days (Figure 1b), the size of the spheres decreased, especially between day seven and 14. In addition to the reason already discussed in the manuscript, it could be explained by the declining

prevention of cell adhesion to the agarose. During the repeated culture medium changes during the sphere cultivation, the initial function of the agarose declined, cells grew out of the spheres and the diameter of the agglomerates decreased. Cell apoptosis cannot be ruled out to be another reason for the decreasing size of the spheres and appears as a physiological process after 14 to 28 days.

The outgrowth of cells out of the microspheres was also recognized by the osteomicrospheres cultivated on glass chamber slides as visualized, showing tissue like behavior by colonizing the adjacent material. In the inner space of the osteomicrospheres, predominantly cubic cells were recognized, similar to the morphology of mature osteoblasts as described in literature (Heinemann et al., 2011). These are cuboidal, 20 to 30 μm wide cells with an oval nucleus, which are organized palisade-like around the matrix they synthesize. Furthermore, these cells are active, expressing extracellular matrix proteins namely collagen type I and osteocalcin as revealed in the present study by immunohistochemistry. The expression of osteonectin confirms the results of other studies demonstrating protein expression at earlier stages of osteoblastic cell lineage differentiation, and being a stimulating factor in the mineralization process. Osteocalcin, one of the latest osteoblast markers, indicates differentiation gradients and the transition to mineral formation (Owen et al., 1990). Although these macromolecules can be found in other tissues, their expression by osteoblasts and deposition into the matrix reflects the biosynthetic repertoire of the osteomicrospheres. In this study, an early and strong osteoblastic differentiation and expression of osteocalcin was detected starting in the core region of the microspheres. The same process was also described for embryonic stem cell micromasses, where the mineralization also started in the core of the spheres after osteogenic differentiation (Handschel et al., 2011). In the present study, osteocalcin expression in the core of the spheres represents beginning mineralization and

emphasizes the tissue-like behavior of these three-dimensional constructs.

The osteomicrosphere's surrounding outer layer of flat elongated cells is similar to that described by Aubin et al. (1998) as elongated and thin bone-lining cells. This cell type is a rather inactive form, contains less cell organelles (Owen et al., 1990) and is typically present on the surface of the bone. In the present study, the cells of the outer layers of osteomicrospheres seem to have an "epithelial" function and they were able to grow out of the cell sphere and to migrate on biomaterial surfaces. The activity of the surface cells and also the core cells was shown by the ability to spread out and grow around biocompatible materials by maintaining their spheroid shape. The fibrin scaffold was chosen for this investigation, because fibrin as a material produced naturally in the body and furthermore is an adequate scaffold material in tissue engineering. The morphological adaptation of the microspheres by spreading of single cells out of the cell complex highlights the biological activity of this tissue-like cell constructs.

Conclusion

The ability to study osteoblasts in an environment close to native tissue is one of the major advantages of the presented micromass culture system. The possibility to assure the reproducible formation of viable osteomicrospheres within a defined period of time that withstand handling procedures may open new paths in extra-corporal bone tissue engineering. Furthermore, the results of the present study suggest the use of the concept as a new test method classified between the conventional monolayer techniques and small animal studies. The cell osteomicrospheres show tissue-like behavior and differentiation performances, the physiological ability to interact with other osteomicrospheres to larger tissue-like complexes and to invade, envelope or encapsulate artificial biomaterials.

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