Formulation and Characterisation of RGD-FBG based Nanoscaffolds as Novel Anti-Cancer drug delivery system.

ABSTRACT:

Aims/Objective: To formulate and evaluate RGD sequenced fibrin/fibrinogen based nanoscaffolds (acid-labile linker) for the treatment of brain tumor targeting to achieve an acid-triggered drug release under a mild acidic condition in tumor.

Place and Duration of Study: Department of Pharmaceutics, Parul Institute of Pharmacy and Research, Parul University, Vadodara, between 2017 to 2021.

Methodology: Nanoscaffolds was prepared with Drug–linker–Fbg conjugate solution, using modified water-in-oil (W/O) emulsification/solvent extraction method. The conjugate solution was constantly injected in soya lecithin (100 ml) using a syringe (1 ml/min) and stirred using Magnetic stirrer for 30 minutes to form stable a W/O emulsion. The present investigation was aimed to evaluate with Particle size, Zeta potential measurement, Percent drug entrapment, Transmission electron microscopy.

Results: As per Evaluation parameters were performed such as Particle size measured as 151.8±2.02 nm to 157.4 ± 3.81 nm. Zeta Potential was found that -37.1±6.32, Percent Drug Entrapment was 91.4±0.37 which indicates that the drug release of lyophilised FBG based nanoscaffolds was indicatively higher as compared to FBG based nanoscaffolds (alone).

Conclusion: As per the study design the formulated nanoscaffolds were found to impart the drug release based on their particle size and percent drug entrapment and hence fights against cancer cells in brain by their EDC-NHS chemistry. Drug of choice Everolimus along with Fbg based nanoscaffolds offers better biocompatibility by exposing dual responsiveness at acidic environment in tunors. The results of in-vitro drug release reveals the better uptake of drug at targeted site of action.

Keywords: *Everolimus, Nanoscaffols, In-vitro drug release.*

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1. INTRODUCTION

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Not all tumors are cancerous; benign tumors do not spread to other parts of the body. Possible signs and symptoms include: a new lump, abnormal bleeding, a prolonged cough, unexplained weight loss, and a change in bowel movements among others. While these symptoms may indicate cancer, they may also occur due to other issues. There are over 100 different known cancers that affect humans. [1-3]

Approximately 5–10% of cancers are due to genetic defects inherited from a person's parents. Cancer can be detected by certain signs and symptoms or screening tests. It is then typically further investigated by medical imaging and confirmed by biopsy. [4-5]

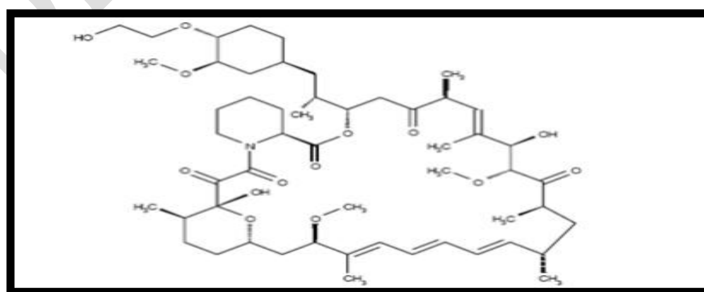
The main brain tumor treatment options may include:

- Radiation therapy (radiotherapy)
- Colloidal techniques
- Surgery
- Biological therapy (targeted drug therapy)
- Hormone therapy
- Chemotherapy.

The current investigation is carried out that aims to treat brain targeted mechanisms by achieving acid triggered drug release under mild acidic condition in tumor.

The drug of selection – Everolimus; inhibitor of mammalian target of rapamycin (mTOR), a serine-threonine kinase, downstream of the PI3K/AKT pathway, inhibits mTOR pathway and hence reduces cell proliferation, angiogenesis that are prominent for cancer. The structural activity relationship of the drug along with the Fibrinogen is known to be dual responsive.

The other anti-cancer drugs include: Carboplatin, Sirolimus, Procarbazine, Bevacizumab etc are used to treat various cancers, however the choice of drug Everolimus is known to have greater affinity towards RGD peptide and has half life of 30 hours (comparatively higher in regards to other anti-cancer agents).



Structure of Everolimus

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1.1 Advantages of Nano-Constructs drug delivery:

1.1.1) As model system to deliver micro molecules as well as macromolecule such as proteins, DNA, etc.

1.1.2) Site specific targeting:

In certain cases Nano-constructs Delivery system with surface attached ligands can bind to target cells ('key and lock' mechanism), or can be delivered into the target tissue by local anatomical conditions such as leaky and badly formed blood vessels, their basal lamina, and capillaries. Examples include anticancer, anti-inflammatory etc.

1.1.3) Site avoidance delivery: Some drugs used in several treatments usually exhibit a narrow therapeutic index causing high toxicity to normal tissues. This toxicity could be minimized by decreasing delivery to normal tissues. Thus the distribution of a variety of anti-neoplastic drugs using Nano-constructs Delivery system formulations can reduce considerably the toxicity to heart, liver or gastrointestinal tract. For example: a) Nano- constructs Delivery system doxorubicin to reduce the cardiac toxicity, and b) Nano- constructs Delivery system amphotericin B to reduce nephrotoxicity.

1.1.4) Sustained / controlled release: Drugs which are rapidly excreted or metabolized 'sawtooth' pattern of plasma drug levels are often observed. Thus the concentration of drug in blood stream oscillates between toxic and sub-therapeutic level, e.g. many antitumour drugs are cleared from blood stream, while same agents, encapsulated in Nano- constructs Delivery system persists in blood for hours. These drug encapsulated Nano- constructs Delivery system as an intravascular sustained release system would be enhanced by increasing life time of circulation and to reduce Nano-constructs Delivery system uptake by fixed phagocyte cells of reticuloendothelial system. The use of Nano- constructs Delivery system sustained release preparation may be of most value for drugs of low therapeutic index. Another application would be the intravascular use of drugs with low water solubility, since these could be maintained in the circulation via Nano- constructs Delivery system encapsulation. Examples are: a) Inhalation of bronchodilator, b) Ocular delivery of antibiotic and c) Topical delivery.

1.1.5) Intravenous delivery of radio imaging agent: Actively or passively targeted Nano- constructs Delivery system can be used as carriers for contrast agents to increase the signal difference between areas of interest and background, and to specifically localize the contrast moieties in the target tissues or organs. The versatility of Nano- constructs Delivery system vesicles to carry different types of compound in the bilayers or in the aqueous compartment makes them suitable for all contrast procedures, including gamma-scintigraphy, magnetic resonance imaging (MRI), computed tomography imaging (CTI), and sonography. Using Nano- constructs Delivery system in diagnostic imaging leads to several advantages, owing to their capability to incorporate multiple contrast moieties, to specifically deliver the agent to the target area, and to enhance the contrasting signal. Scintigraphic techniques using 99mTc, are useful tools for the noninvasive analysis of the in vivo behaviour of Nano- constructs Delivery system. Using these techniques, quantitative information regarding the in vivo movement, distribution, and fate of the Nano- constructs Delivery system becomes readily available.

1.1.6) Gene therapy: Conventional Nano- constructs Delivery system have also been tried as delivery system to deliver DNA into cells. The rationality was the ability of Nano- constructs Delivery system to enhance intracellular accumulation i.e. facilitate transfer of these large and heavily changed molecules across rather impermeable cell membrane. Cationic Nano- constructs Delivery system are the most suitable transfecting vectors. Gene encapsulation in Nano- constructs Delivery system vesicles allows condensation of DNA plasmid into a structure, and protects DNA against degradation

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during storage and in the systemic circulation of the gene encoding a therapeutic protein. Moreover, structural organization of the gene-delivery system must bypass the cell membrane and facilitate escape, avoiding DNA degradation in lysosomal compartment.

1.1.7) Now the herbal products like flavonoids are given orally through Nano-constructs Delivery system delivery system. These herbal products containing Nano-constructs Delivery system are known as harbosomes.

1.1.8) Nano- constructs Delivery system also improved transfer of hydrophilic and charged molecules such as chelators, antibiotics, plasmids and genes into cells.[6-11]

1.2 RGD-FBG Targeting :

The RGD (Arginine-Glycine-Aspartate) sequenced fibrin/fibrinogen based nanoscaffolds specifically binds to the target moiety expressed mainly in the cancerous cells and not in the normal body cells, and release the drug at the cancer site, thereby providing a site-specific action.

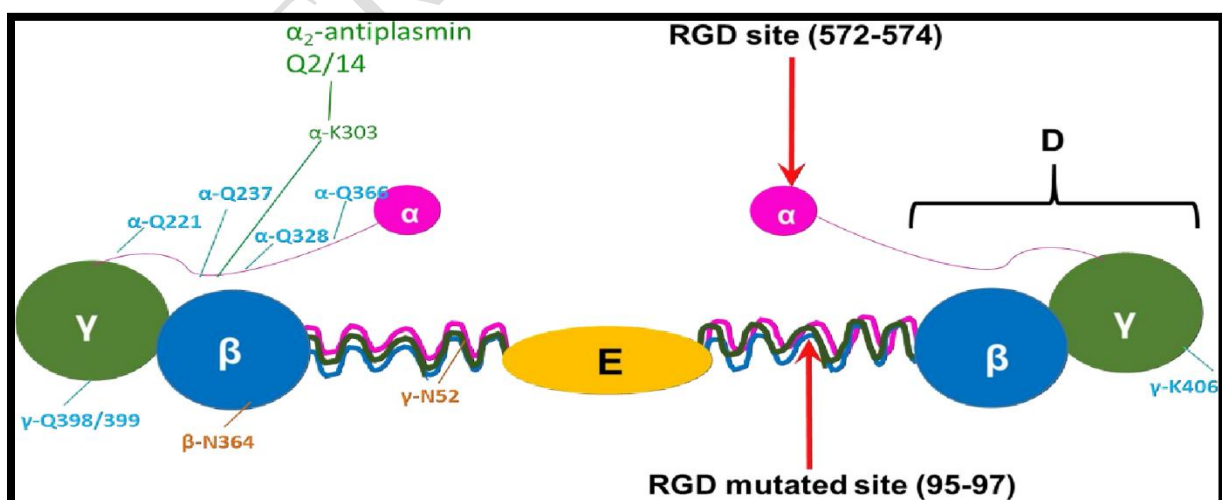
The RGD sequenced fibrin/fibrinogen based nanoscaffolds (acid-labile linker) is known to achieve an acid-triggered drug release under a mild acidic condition in tumor.

Therefore, the biocompatibility of Fbg and the RGD sequence present in Fbg may offer a great potential for use in developing biocompatible drug delivery carriers as well as for targeting cancer cells.

Fbg possesses tumor targeting property through its arginine-glycine-aspartate (RGD) peptide sequence.

RGD peptide, well-known as a tumor targeting peptide, has high affinity for αvß3 integrin, which is over expressed on the surface of various cancer cells.

Based on the specific interaction between RGD peptide and an integrin, RGD peptide can be extensively conjugated to various drug carriers, showing enhanced cellular uptake by tumor cells. The structural relationship of RGD-FBG was presented as below -



2. MATERIALS AND METHODS:

2.1 Materials:

Everolimus, pharmaceutical grade material was received as research sample from Alembic Pharmaceuticals, Hyderabad. Fibrinogen, Acetonitrile, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC or EDAC), Eagle minimum essential medium (EMEM), Fetal Bovine Serum, Non-essential Amino Acids (NEAA), Sodium pyruvate, Glutamine, Hank's Balanced salt solution were purchased from sigma Aldrich, Vadodara. 3-maleimidopropionic acid hydrazonium trifluoroacetic acid (3-MAH) was purchased from Roquette, Mumbai. 0.1 M Phosphate Buffer Saline (PBS), Acetone and N –hydroxysulfosuccinimide (NHS) were purchased from Merck, Mumbai. [12-15]

2.2 MEHODOLOGY:

2.2.1 PRE-FORMULATION STUDIES:

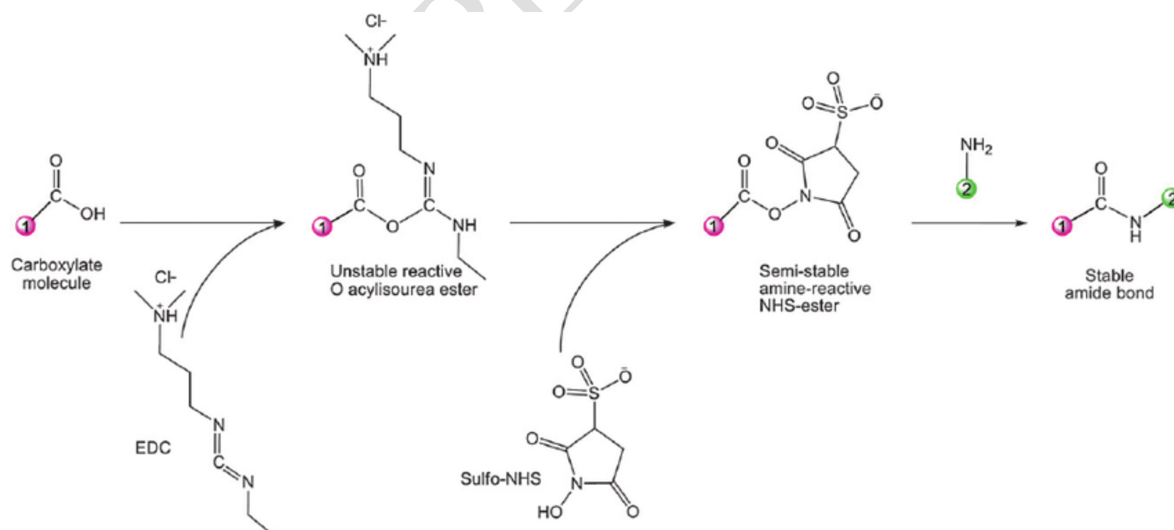
Everolimus drug was observed visually for the color, odor, and solubility study was carried out.

2.2.2 SOLUBILITY STUDY: Solubility tests was carried out quantitatively. Initially, water and acetonitrile were used to assess the solubility of drugs. 10 mg of the drug sample was taken in clean dry test tubes, gradually the acetonitrile (solvent) was added in aliquots of 0.1 ml increment with constant shaking until the drug is dissolved completely. **[16-17]** The amount of solvent required to solubilize the drug sample was noted and the solubility of drugs was compared with the reported solubility values.

2.2.3 CHEMISTRY OF EDC-NHS:

2.2.3.1 EDC (or EDAC; 1-ethyl-3-(3-dimethylaminopropyl), carbodiimide hydrochloride)

2.2.3.2 It is a zero-length cross-linker, that does not require introduction of spacer molecule thus, it mediates conjugation of two molecules by forming a bond with no additional atoms, hence one atom is covalently attached to other with no intervening linker, the chemistry of EDC-NHS is shown is figure 1 as depicted below:



where 1=Hydroxy group, 2=Amide bond

Figure 1. Chemistry of EDC-NHS

2.2.3.3 EDC reacts with carboxylic acid groups to form an active O-acylisourea intermediate that is easily displaced by nucleophilic attack from primary amino groups in the reaction mixture. [18]

2.2.3.4 The primary amine forms an amide bond with the original carboxyl group, and an EDC by-product is released as a soluble urea derivative. The O-acylisourea intermediate is unstable in

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aqueous solutions; failure to react with an amine results in hydrolysis of the intermediate, regeneration of the carboxyls, and the release of an N-unsubstituted urea.

2.2.3.5 N-hydroxysuccinimide (NHS) or its water-soluble analog (Sulfo-NHS) is often included in EDC coupling protocols to improve coupling efficiency. EDC couples NHS to carboxyl, forming an NHS ester that is considerably more stable than the O-acylisourea intermediate while allowing for efficient conjugation to primary amines at physiologic pH.

2.2.4 METHOD OF SYNTHESIS:

STEP 1: Fbg was dissolved at concentration 10 mg/ml in 0.1 m sodium phosphate buffer (ph 7.4), along with 2-iminothiolane (2-it). stirred for one hour. Everolimus (drug) was dissolved in above buffer, and then EDC:NHS: 3-mah (5:2:1) was added. Drug mixture was added to above solution containing thiolyted fibrinogen. Reaction of entire composition of drug conjugate mixture for 2 hours at room temperature was performed, (if) turbidity appears, removed via centrifugation/filtration. The conjugate was purified by dialysis using 0.01 m sodium phosphate buffer, and the conjugation was measured in IR.

2.2.5 METHOD OF FORMULATION: [20]

2.2.5.1 Nanoscaffolds was prepared with Drug–linker–Fbg conjugate solution, using modified water-in-oil (W/O) emulsification/solvent extraction method.

2.2.5.2 The conjugate solution was constantly injected in soya lecithin (100 ml) using a syringe (1 ml/min), and stirred using stirrer for 30 minutes to form stable a W/O emulsion.

2.2.5.3 Then, the emulsified solution was gradually heated up to 60°C for 1 hour using a hot plate. Acetone was added slowly to extract the solvent and evaporate the water.

2.2.5.4 Once the aqueous phase was evaporated, solid nanoscaffolds were formed after continuous addition of acetone.

2.2.5.5 The solution was stirred for another 10 minutes to remove any residual solvent. the nanoscaffolds were collected by centrifugation at 20,000 rpm for 30 minutes.

2.2.6: Transmission Electron Microscopy (TEM):

The morphology of Nanoscaffolds was examined using transmission electron microscopy JEOL JEM-2100 HR. Nanoscaffolds was visualized after staining with phosphotungstic acid on a copper grid under TEM. Photograph of TEM images of optimized batch B_{13} are shown in figure 2.

2.2.7 Particle Size and Measurement: Particle size and zeta potential of prepared nanoscaffolds was measured by dynamic light scattering using Malvern Zetasizer.

2.2.8 Zeta Potential Measurement:

Zeta Potential was determined by using zeta sizer (Malvern instruments, UK) using clear disposable zeta cell by employing field strength of 20 V/cm. Dispersant medium was used water and set the dispersant reflective index will be lipid, the electrophoretic mobility will be converted into to the zeta potential via Helmholtz smoluchowski equation.

2.2.9 Percent Drug Entrapment (PDE):

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Required quantities of nanoscaffolds were taken into a test tube and added into the solvent medium. Test tube was allowed to centrifuge to separate free drug and entrap drug. Drug was analyzed by UV spectrophotometer. From the free drug content encapsulation efficiency was calculated from the following equation.

- % Encapsulation Efficiency = Total drug loading – Free drug/ Total drug loading *100

3.0 RESULTS AND DISCUSSIONS:

3.1 Pre-formulation studies

3.1.1 Appearance and odor:

Everolimus is yellowish powder with no characteristic odor as compared with reported color that is slightly beige odor.

Discussion: The appearance and odor of the drug complies with the reported appearance and odor.

3.1.2 Solubility study: Solubility tests was carried out quantitatively as a test for purity. The results of qualitative solubility study shown in **Table 1.**

Table 1: Results of quantitaive solubility study.

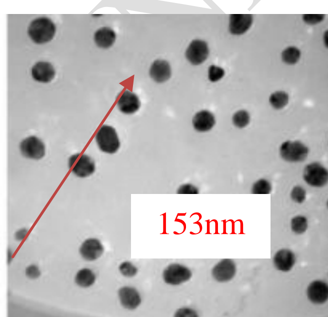
Drugs	Observed solubility	Reported solubility
Everolimus	Soluble in Ethanol, DMSO, DMF, sparingly soluble in aqueous buffer.	~0.00163 mg/ml in ethanol

Discussion: The solubility of the drug complies with the reported values.

3.3 Characterization of Fbg Nanoscaffolds:

3.3.1 Transmission electron microscopy (TEM):

The morphology of the particles was studied using a Transmission electron microscope (TEM). It was performed with 1 % phototungustic acid. TEM images of the formulated nanoscaffolds with range 153nm and 303nm are shown here as below:



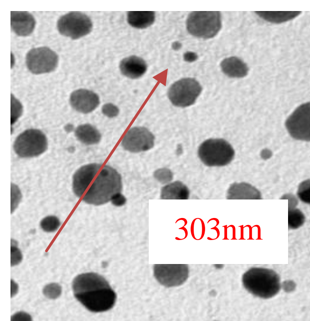


Figure 2: TEM Images of Nanoscaffolds

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Results:

TEM study revealed that particle size of nanoscaffolds was found to be in the range of 10-1000nm. Particles of nanoscaffolds were found to be spherical in shape.

3.3.2 Particle Size and Measurement, Zeta Potential and PDE was measured as results reflected in Table 2.

Table 2: Results of evaluation of optimized batch:

Batch	Particle size (nm)	PDE Mean±SD	Zeta potential (mV)
F14	151.8±2.02	91.4±0.37	-37.1±6.32

Discussion: Particle size of the prepared nanoscaffolds was measured by zetasizer.

Particle size for optimized batch was found to be *151.8±2.02*. Percentage drug entrapment was found 91.4±0.37 and zeta potential was found to be -37.1±6.32 mV.

3.3.3 In-vitro drug release study:

The evaluation was performed using dialysis bag with a receiver compartment volume of about 20 ml with continuous stirring on magnetic stirrer which was kept at a temperature of 37 ± 0.5°C. Result of % cumulative drug release (%CDR) and graph of release profile as shown below for nanoscaffolds was obtained:

Table 3: Drug Release Assay

Donor compound	**Fbg based Nanoscaffolds**
Diffusion media	Phosphate Buffer 5.5
Sampling time interval	Initial, 2,3,4,5,6,7,8,24 hours
Analysis by UV Spectrophotometer	277 nm

Table 4: %CDR of Fbg based Nanoscaffolds and Lyophilised Nanoscaffolds

Time (hrs)	%CDR of Fbg based Nanoscaffolds	%CDR of Lyophilised Nanoscaffolds
1	1.45 ± 0.23	1.15 ± 0.18
2	3.91 ± 0.15	2.25 ± 0.13
3	9.25 ± 0.12	9.03 ± 0.11
4	13.30 ± 0.10	12.43 ± 0.12
5	19.73 ± 0.21	18.98 ± 0.20

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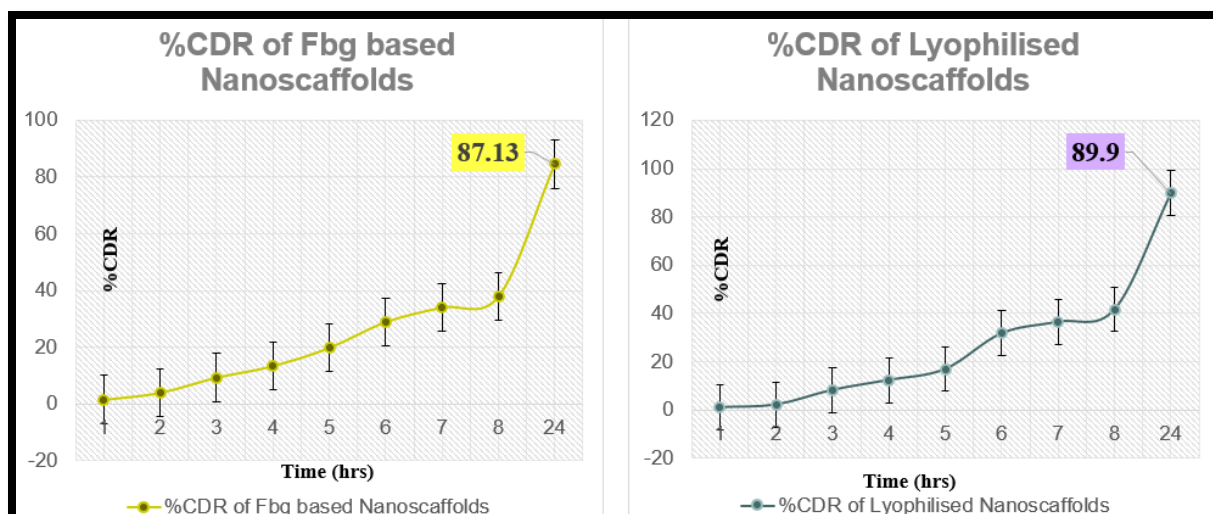
6	28.81 ± 0.58	27.79 ± 0.53
7	33.96 ± 0.26	34.55 ± 0.28
8	37.82 ± 0.13	38.72 ± 0.14
24	87.13 ± 0.41	89.90 ± 0.51

(Where n=3, Mean ± SD)



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Results of Drug Release up to 24 Hrs:

- Fbg based Nanoscaffolds (Everolimus) was found to be 87.13±0.41% up to 24 hrs
- Lyophilized Fbg based Nanoscaffolds (Everolimus) was found to be 89.90 ± 0.51% up to 24 hrs

4.0 Conclusion:

The Nanoscaffolds formulated using RGD-sequence were observed that may treat glioblastomas as they are found to degrade at acidic pH (4-6), acid labile linkers were specifically designed to remain stable at the neutral pH of blood circulation, but undergo hydrolysis and release the cytotoxic drug in the acidic environment of the cellular compartments, RGD peptide is well-known as a tumor targeting peptide, has high affinity for $\alpha_v ß_3$ integrin, which is over expressed on the surface of various cancer cells.

The results of evaluation study reveal the particle size 151.8±2.02, zeta potential 37.1±6.32 mV and percent drug entrapment 91.4±0.37 to be 10-fold higher in their lyophilized form as compare to Fbg used individually.

The in-vitro drug release of study performed up-to 24 hours shows drug release of nanoscaffolds in its

lyophilized state was higher as compared to non-lyophilised state, hence the drug uptake is comparatively higher and effective at its targeted site of action.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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